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Expression of the *Saccharomyces cerevisiae* Succinate Dehydrogenase in *Escherichia coli*

by

Jennifer L. Douglas ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Master of Science

Department of Biochemistry

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Abstract

We developed an expression system to functionally overproduce the *Saccharomyces cerevisiae* mitochondrial succinate dehydrogenase (SDH) enzyme complex in *Escherichia coli* in order to facilitate biophysical and mutational studies. We designed and constructed a synthetic SDH operon based on the *E. coli* fumarate reducatase (*frd*) operon, which encoded all four mature yeast subunits modified for expression in *E. coli* . Anaerobic expression of the yeast SDH operon under control of the *frd* promoter was tested with antisera against three of the four subunits, while aerobic expression of the operon under the T7 promoter was additionally detected by radiolabeling. Immunoreactive proteins were detected with antisera against the Sdh1p, Sdh2p, and Sdh3p subunits. However, the yeast SDH operon failed to produce assembled, functional enzyme. Further experiments to improve overproduction by the synthetic yeast SDH operon in *E. coli* are discussed.

Table of Contents

Chapter I: Introduction **1**

I-1. Introduction 2

I-2. The Electron Transport Chain of Mitochondria 2

I-3. Yeast SDH as a Model System 4

I-4. Structure and Function of SDH and FRD 4
    I-4.a) The Enzyme Complex 4
    I-4.b) The Flavoprotein 5
    I-4.c) The Iron-Sulfur Subunit 6
    I-4.d) The Anchor Subunits 8

I-5. Assembly of SDH and FRD 10
    I-5.a) Order of Subunit Assembly 10
    I-5.b) Insertion of Cofactors 11
    I-5.c) Proteolytic Processing During Assembly 12
    I-5.d) Accessory Folding Proteins 13

I-6. Genes Encoding SDH and FRD 14
    I-6.a) Eucaryotic SDH/FRD Genes 14
    I-6.b) Procaryotic SDH/FRD Genes 15
    I-6.c) Regulation of SDH and FRD Gene Expression 16

I-7. Thesis Objective 18

Chapter II: MATERIALS AND METHODS **24**

II-1. Strains and Plasmids 25

II-2. Growth Media 25

II-3. General DNA Isolation, Purification, and Cloning Techniques 26

II-4. Di-/Trinucleotide Sticky End Cloning (Di/Trisec) 27

II-5. Polymerase Chain Reaction 27

II-6. Oligonucleotide Synthesis 28

II-7. Construction of the Synthetic Yeast SDH Operon 28
    II-7.a) Isolation of the Yeast SDH Genes 28
    II-7.b) Construction of Fragment Dimers 29
    II-7.c) Cloning the Gene Dimers 30
    II-7.d) Construction of the Synthetic Yeast SDH Operon From Cloned Dimers 30

II-8. Modifications To Correct the Synthetic Yeast SDH Operon: 31
    II-8.a) Replacement of an Internal NgoM I/EcoR I fragment in the SDHI gene 31
    II-8.b) Correction of Dimer 2 of the Synthetic Yeast SDH Operon 31

II-9. Sequencing of the Synthetic Yeast SDH Operon (Plasmid pSdhOpF) 32

II-10. Construction of Plasmid pSdhOpT7: Placing the Synthetic Yeast SDH Operon Under the Control of the T7 Promoter 32

II-11. Expression Studies 33
  II-11.a) Anaerobic Expression of the Synthetic Yeast SDH Operon From the Frd Promoter 33
  II-11.b) Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter: 34
    II-11.b) i. Selective Radiolabeling of Plasmid-Encoded Proteins 34
    II-11.b) ii. Expression of Non-Radiolabeled, Plasmid-Encoded Proteins 34

II-12. Preparation of Membrane and Soluble Cellular Fractions 35

II-13. Determination of Protein Concentration 35

II-14. Isolation of Mitochondria from Yeast 35

II-15. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 36
  II-15.a) Laemmli SDS-PAGE 36
  II-15.b) Tricine SDS-PAGE 36

II-16. Western Blot Anaylsis 36

II-17. Primary Antibodies 37

II-18. Audoradiography Analysis 37

Chapter III: Expression of Yeast SDH in *E. coli* 52

III-1. Introduction 53

III-2. Detailed Description of the Synthetic Yeast SDH Operon 54

Chapter IV: Results 66

IV-1. Introduction 67

IV-2. Construction of the Synthetic Yeast SDH Operon 67

IV-3. Expression Studies 67
  IV-3.a) Anaerobic Expression of the Synthetic Yeast SDH Operon From the frd Promoter 67
  IV-3.b) Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 RNA Polymerase Promoter 70

Chapter V: Discussion 85

V-1. Discussion 86

V-2. Future Studies 95

Bibliography 98

List of Tables

TABLE II-1: Strains Used In This Study 38

TABLE II-2: Plasmids and Phage Used in This Study 39

TABLE II-3: PCR Amplification of the SDH Operon Fragments 40

TABLE II-4: Sequence of Primers 41

TABLE II-5: PCR Amplification of Dimers 42

List of Figures

Figure I-1. Schematic Representation of the Mitochondrial Electron Transport Chain 20

Figure I-2. Structure of SDH 21

Figure I-3. Molecular Structure of the [2Fe-2S], [3Fe-S], and [4Fe-S] Iron-Sulfur Clusters 22

Figure I-4. Regulatory Scheme for Oxygen and Nitrate Control of *E. coli sdh* and *frd* operon Gene Expression 23

Figure II-1. Construction of the Synthetic Yeast SDH Operon 43

Figure II-2. Modification of the Synthetic Yeast SDH operon in plasmid pBSd123 to Reduce Sequencing. 46

Figure II-3. Modification of the Synthetic Yeast SDH Operon in plasmid p123R to Correct Dimer 2. 48

Figure II-4. Construction of plasmid pSdhOpT7: Placing the Synthetic Yeast SDH Operon Under the Control of the T7 Promoter 50

Figure III-1. Organization of the *E. coli frd* Operon and the Synthetic Yeast SDH Operon 58

Figure III-2. Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 1-26 59

Figure III-3. Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 775-841 60

Figure III-4. Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 2603-2667 61

Figure III-5: Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 3366-3430 62

Figure III-6: Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 3833-3899 63

Figure III-7: Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 4295-4334 64

Figure III-8: Detailed Nucleotide Sequence of the Synthetic Yeast SDH Operon at the Junctions Between the SDH and FRD-derived DNA: nucleotides 5067-5090 65

Figure IV-1. Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh1p Antiserum 75

Figure IV-2. Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh2p Antiserum 76

Figure IV-3. Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh3p Antiserum 77

Figure IV-4. Western Blot Analysis Using the Sdh1p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli.* 78

Figure IV-5. Western Blot Analysis Using the Sdh2p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli.* 79

Figure IV-6. Western Blot Analysis Using the Sdh3p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli.* 80

Figure IV-7. Autoradiogram of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter 81

Figure IV-8. Western Blot Analysis Using Sdh1p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter 82

Figure IV-9. Western Blot Analysis Using Sdh2p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter 83

Figure IV-10. Western Blot Analysis Using Sdh3p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter 84

ABBREVIATIONS AND NOMENCLATURE

ADP	adenosine diphosphate
amp	ampicillin
ATP	adenosine triphosphate
BSA	bovine serum albumin
cam	chloramphenicol
Cys	cysteine
cyt	cytochrome
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
Di/Trisec	Di-/Trinucleotide sticky end cloning
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetate
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
Fe-S	non-heme iron and acid labile sulfur group
FMN	flavin mononucleotide
FNR	fumarate nitrate regulator
FRD	fumarate reductase
g	standard acceleration of gravity (9.81 m/s^2)
GF	glycerol fumarate
6-HDNO	6-hydroxy-D-nicotine oxidase
IPTG	isopropyl-ß-D-thiogalactopyranoside
kan	kanamycin
kbp	kilobase pairs
kDa	kilodaltons

LB	Luria-Bertani broth
mL	milliliter
mRNA	messenger RNA
mV	millivolt
nm	nanometer
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
P_i	inorganic phosphate
PMSF	phenylmethanesulfonyl fluoride
psi	pounds per square inch
PVDF	polyvinylidene difluoride
Q	quinone
QH_2	quinol
QH•	semiquinone
RBS	ribosomal binding site
RNA	ribonucleic acid
SAP	shrimp alkaline phosphatase
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTR	untranslated region
wt	wildtype
µCi	micro Curie
°C	degree (s) Celsius

Chapter I: Introduction

I-1. Introduction

Succinate dehydrogenase (SDH) is a membrane-bound, multisubunit enzyme that is essential for both the Krebs cycle and the respiratory electron transport chain. SDH, also known as Complex II of the respiratory chain, or succinate:quinone reductase (SQR), catalyses the oxidation of succinate to fumarate as part of the Krebs cycle, and passes the reducing equivalents on to ubiquinone.

The enzyme generally consists of four distinct cofactor-containing subunits. The two hydrophobic subunits form a membrane anchor for the two soluble, catalytic subunits. The complex is located in the inner membrane of mitochondria in eucaryotes, or in the plasma membrane in procaryotes. SDH is structurally and functionally related to fumarate reductase (FRD). Fumarate reductase, also referred to as quinol:fumarate reductase (QFR), is found in anaerobic and some facultative organisms respiring with fumarate as the terminal electron acceptor. Both enzymes reversibly interconvert succinate and fumarate coupled to electron transfer to or from quinone, but they have different substrate affinities and reaction rates (60).

SDH is a very attractive model for studying the structure, function, and biogenesis of a multisubunit membrane-bound, mitochondrial respiratory complex. Structurally, the enzyme contains several electron transporting cofactors including a covalent flavin and multiple iron-sulfur clusters. SDH is the simplest member of the mitochondrial respiratory complexes, being composed of only four nuclear-encoded subunits. In addition, SDH is very well conserved between eucaryotes and procaryotes and presents a good model for understanding electron transport.

The research in the Lemire laboratory is focused on the structure, function, and biogenesis of the yeast mitochondrial SDH. The goal of my thesis work was to design and construct an *E. coli* expression system for the yeast SDH. The aim of this introduction is to present an overview of our current knowledge on the SDH and FRD family of enzymes, focusing on the structure, function, expression, and assembly into complexes.

I-2. The Electron Transport Chain of Mitochondria

Mitochondria are subcellular organelles present in most eucaryotic cells and are defined by two highly specialized membranes. They are proposed to have originated as procaryotes that established a symbiotic relationship with an ancestral precursor of the eucaryotic cell. This organelle is the 'power house' of eucaryotic cells and is responsible for carrying out aerobic respiration, oxidative phosphorylation, and fatty acid metabolism. In procaryotes, the respiratory assemblies and the ATP-synthesis complex are located in the plasma membrane. Respiration and oxidative phosphorylation are coupled processes that provide the cell with the major source of useable energy. The overall process requires a series of soluble enzymes and several membrane complexes, which constitute the electron transport chain (Figure I-1). Complexes of the electron transport chain (Complexes I, II, III, and IV) and oxidative phosphorylation (Complex V) are embedded in the inner mitochondrial membrane. Electrons are transferred down the chain from complex to complex and then finally to oxygen. The transfer of electrons through the respiratory protein assemblies occurs via redox groups such as flavins, iron-sulfur clusters, heme, and copper ions. Electron transport between complexes occurs either via a mobile, lipid soluble carrier, ubiquinone, or a single cytochrome c. As the electrons are passed down through the electron transport chain in a step-wise controlled manner, the energy that is released is harnessed to pump protons from the matrix out to the intermembrane space creating an electrochemical gradient across the inner membrane, which is used to power ATP synthesis. Complex II (SDH) is not a coupling site in the respiratory chain (Figure I-1).

The two highly specialized membranes of mitochondria are crucial to the organelle's cellular function. The inner membrane is highly folded into a series of internal ridges called cristae to increase the surface area. This membrane is ion-impermeable and has one of the highest protein:lipid ratios. The inner membrane encloses the matrix compartment, which houses enzymes involved in the Krebs cycle and fatty acid metabolism. A second, chemically distinct compartment, the intermembrane space, is bound by the inner and outer mitochondrial membranes. The outer membrane, in contrast to the inner membrane, is permeable to most small molecular ions because it contains many copies of porin, a transmembrane protein that forms a large pore. Several additional proteins are present for the transport of molecules larger than 10 kDa.

Mitochondria have their own genome and are capable of synthesizing proteins in the matrix. The mitochondrial genome only encodes approximately 1 % of mitochondrial proteins. Among these proteins are thirteen subunits that are synthesized in the matrix and are then assembled as components of Complexes I, III, IV and V (3, 22). Significantly, Complex II (SDH) is entirely nuclear-encoded in almost all eucaryotes.

The assembly of the mitochondrial multisubunit proteins is a fascinatingly intricate process. Biogenesis of these complexes involves the coordination of two genomes, the targeting and import of cytosolically synthesized proteins, proteolytic post-translational processing, cofactor insertion, assembly, and the correct insertion into the appropriate membrane. SDH undergoes all of these processes and presents an excellent model for studying biogenesis of mitochondrial membrane bound protein complexes..

I-3. Yeast SDH as a Model System

The SDH enzymes of bovine heart mitochondria, *E. coli*, *B. subtilis*, and *S. cerevisiae*, and the FRD enzymes of *E. coli* and *Wollinella succinogenes* have been extensively studied. *Saccharomyces cerevisae* offers a number of significant genetic advantages as a eucaryotic model system for investigating the assembly pathway of mitochondrial complexes. It is currently the only mitochondrial complex of the SDH/FRD family of enzymes that can be studied by site-directed mutational experiments. Yeast is a facultative anaerobe that is able to meet its energy needs by substrate level phosphorylation without oxidative phosphorylation (79). A mutant, non-functional respiratory chain is lethal in most other eucaryotic organisms. This metabolic feature of yeast makes it possible to carry out mutational studies of SDH that are not possible in other aerobic organisms. Furthermore, genetic recombination in yeast generally occurs via homologous recombination, which makes it easy to create nuclear disruption mutants of targeted genes (117). Genetic studies of SDH can be performed by creating knock-out mutants, complementing the mutant cells with wildtype or modified genes carried on a plasmid, and characterizing the resulting phenotypes.

Yeast SDH is produced in quantities too low to easily enable purification of the complex. In the Lemire lab, attempts to overproduce SDH in yeast failed to produce functional assembled enzyme. Therefore, we decided to overexpress it in *E. coli*. The yeast model system would benefit from the ability to produce large quantities of purified, functional protein for biochemical and mutational studies.

I-4. Structure and Function of SDH and FRD

I-4.a) The Enzyme Complex

Succinate dehydrogenase (SDH) and fumarate reductase (FRD) are enzyme complexes similar in both structure and function (2). *In vivo*, SDH is found in aerobically respiring organisms and catalyses the oxidation of succinate to fumarate coupled to the reduction of quinone, while FRD is found in anaerobically respiring organisms and catalyses the reduction of fumarate to succinate coupled to the oxidation of quinols. Both enzymes are membrane-bound, cofactor-containing complexes generally composed of four non-identical subunits (Figure I-2). In yeast SDH, the four subunits are named Sdh1p, Sdh2p, Sdh3p, and Sdh4p in order of decreasing size. For the *E. coli* complex, the four subunits are named FrdA, FrdB, FrdC, and FrdD in order of decreasing size. The catalytic dimer, exposed to the cytoplasm in procaryotes or to the mitochondrial matrix in eucaryotes, consists of a flavoprotein (Sdh1p or FrdA; about 70 kDa) with a covalently attached FAD, and an iron-sulfur protein (Sdh2p or FrdB; about 27 kDa) containing three iron-sulfur clusters. The anchor domain, that functions to both bind the soluble catalytic dimer to the membrane and confer reactivity with quinones, is usually composed of one or two membrane proteins (Sdh3p or FrdC; about 17 kDa, and Sdh4p or FrdD; about 13 kDa) that are often associated with a *b*-type heme prosthetic group. The structure and functions of each of the subunits is discussed below.

I-4.b) The Flavoprotein

The largest subunit of 70 kDa contains both the covalently linked FAD cofactor and the substrate binding site. It is often referred to as the flavoprotein (Fp) subunit. The amino acid sequence of the flavoprotein subunits are well conserved among various sources (2).

Succinate, fumarate, and malate are dicarboxylates that are substrates for the binding site, while malonate and oxaloacetate are potent competitive inhibitors of the site (2). The enzyme complex exhibits different affinities for its substrates and inhibitors depending on whether FAD is in an oxidized or a reduced state (1, 78). This implies that the redox state of the FAD cofactor affects the structure of the substrate binding site and that the FAD is likely situated in close proximity to the substrate binding site (2). The specific residues of the active site and the mechanism of substrate binding are not fully understood. Some key residues in three highly conserved regions have been identified as being near or part of the binding site in studies using chemical modification of substrate protectable residues.

The covalently bound FAD is one of the prosthetic redox groups that forms part of the electron transport pathway within SDH/FRD. It is the first electron acceptor during oxidation of succinate, or the last electron donor during fumarate reduction. FAD is a two electron carrier unlike the other prosthetic groups in the SDH/FRD enzymes, which are one electron carriers. It has an intermediate semiquinone state (FADH•) that allows it to accept two electrons and then donate them one at a time to the next carrier, thus forming a bridge between one and two electron carriers. The FAD is covalently bound to the Fp by an 8α-N(3)-histidyl-FAD linkage at a conserved histidine residue (145). The histidine resides within a highly conserved region of the SDH/FRD Fp, however, the sequence surrounding the histidine residue is not conserved in other non-SDH/FRD 8α-N(3)-histidyl-FAD containing enzymes suggesting the sequence is required for enzyme activity not FAD-binding (46). Non-covalent interactions are also present between the Fp and FAD. In particular, two conserved regions are predicted to form a binding-pocket around the adenosine monophosphate portion of FAD (2, 151).

An interesting question that has still not been resolved despite many investigations is the mechanism of covalent flavin attachment. There is mounting evidence to support an autocatalytic mechanism as opposed to an enzyme mediated process. The flavoprotein, 6-hydroxy-D-nicotine oxidase (6-HDNO) from *Arthrobacter oxidans,* also covalently binds FAD at a histidine residue by an 8α-N(3)-histidyl flavin linkage. When expressed in *E. coli*, the majority of 6-HDNO produced contains covalently linked FAD (12). Furthermore, purified apoenzyme can be flavinylated *in vitro*. Together, the evidence suggests that covalent attachment of FAD to 6-HDNO is autocatalytic (14). In contrast, the *Bacillis subtilis* SDH was expressed in *E. coli* as an apoenzyme (54). A possible explaination for this is that the mechanism of covalent flavinylation is not self-catalytic and requires *B. subtilis* accessory proteins.

In yeast, the SDH Fp (Sdh1p) is flavinylated in the mitochondrial matrix following import and the removal of the targeting presequence (111). Citric acid cycle intermediates are allosteric effectors that stimulate FAD insertion in SDH/FRD (11, 111). In yeast, it was found that the iron-sulfur subunit also stimulated, but was not required for flavinylation (111). Both the allosteric effectors and the iron-sulfur subunit are suggested to stimulate flavinylation by allowing the Fp to attain a conformation needed for flavinylation.

The role of covalent flavin attachment in the SDH/FRD enzymes is still unclear. In yeast, the covalent attachment is not necessary for the assembly of the complex (112). It has been observed, however, that the covalent attachment of FAD is required for succinate oxidation activity. A possible role for the covalent bond may be to raise the FAD redox potential to optimize the enzyme's catalytic potential. Mutants have been isolated for *E. coli* FRD and yeast SDH that are only capable of binding FAD non-covalently (9, 110). These enzymes retain the ability to reduce fumarate but have lost succinate oxidation activity (9, 110). *Desulfovibrio multispirans* FRD contains a non-covalently bound FAD and is not able to oxidize succinate (52). SDH from *Mycobacterium phlei*, which may bind FAD non-covalently, still has succinate dehydrogenase activity (108).

I-4.c) The Iron-Sulfur Subunit

The 30 kDa subunit harbours three iron-sulfur clusters (Fe-S) and is often referred to as the iron-sulfur protein (Ip). The Ip, like the Fp, from various organisms is very well conserved. It is intimately associated with the Fp and acts as a bridge and conductor of electrons from the flavin cofactor to the membrane anchor proteins.

Fe-S clusters in iron-sulfur proteins are important in a wide range of oxidation/reduction reactions in biological systems. Several types of Fe-S clusters are known. All SDH/FRD enzymes have similar iron-sulfur cluster compositions with the exception of the FRD of yeast. The yeast FRD is composed of a single subunit and does not contain any iron-sulfur clusters (100). The iron-sulfur clusters of SDH/FRD are named Center 1 ([2Fe-2S]), Center 2 ([4Fe-4S]), and Center 3 ([3Fe-4S]) and are responsible for mediating single electron transport within the subunit (Figure I-3). Each iron-sulfur cluster has a characteristic redox potential. The redox potentials of each cluster are determined both by its intrinsic properties and by the protein environment that surrounds the redox center allowing them to be used for a variety of reactions (82). Almost all iron-sulfur clusters are liganded to the polypeptide chain by covalent linkage to the sulfhydryl groups of cysteine residues. A number of consensus sequences have been suggested for identifying cysteine residues involved in the ligation of Fe-S clusters. Generally, they consist of three closely spaced Cys residues and a distant Cys residue as the final ligand. The Ip subunit contains three groups of Cys residues, which are thought to be iron-sulfur cluster ligands. A number of mutational studies have been performed on the *E. coli* and the *B. subtilis* Ips to identify important residues. The [2Fe-2S] and [3Fe-4S] clusters can be assembled in the absence of the Fp subunit unlike the [4Fe-4S] group suggesting that the [4Fe-4S] cluster may have a ligand provided by the Fp (69). One of the ligands for the [2Fe-2S] cluster may be provided by an aspartate residue instead of a cysteine residue in the *E. coli* SDH (148). This may be responsible for the exceptionally high redox potential observed for the [2Fe-2S] iron-sulfur centers of the SDH/FRD family of enzymes.

Information about the structure and spatial organization of the iron-sulfur clusters has been obtained from EPR-detectable magnetic spin-spin interactions studies. The [2Fe-2S] cluster is located in the N-terminal domain of the Ip and is the first electron acceptor after flavin. It is evolutionarily related to the [2Fe-2S] cluster in plant ferredoxins. The [4Fe-4S] and [3Fe-4S] clusters are located close together in the C-terminal domain of the Ip. The [4Fe-4S] cluster has a low potential and may not be directly involved in electron transfer. Instead, it may be important for modifying the neighboring clusters (121). Alternatively, the apparent low potential detected may be an artifact of anticooperative interactions between the redox centers (120). As one of the coordinating ligands may be provided by the Fp subunit, this Center may provide a structural bridge between the two subunits (2). The [3Fe-4S] cluster transfers electrons to/from quinone, and is situated close to the quinone binding sites (20). The distances between the FAD and [2Fe-2S] cluster are 12-18 Å, the [4Fe-4S] and [3Fe-4S] clusters are 9-12 Å apart, whereas the [2Fe-2S] and [3Fe-3S] clusters are estimated to be 10-20 Å apart.

The mechanism of the assembly of iron-sulfur clusters is not understood. It is not known whether cluster formation occurs via an autocatalytic or an enzyme-mediated process. The iron-sulfur clusters of SDH/FRD can form independently of each other, as shown by the assembly of [2Fe-2S] in an *E. coli* FRD Ip C-terminal deletion mutant (69). *E. coli* FRD Ip expressed in the absence of the Fp subunit contains assembled Centers 1 and 3, suggesting that the iron-sulfur centers are inserted before the Ip associates with the Fp (69). The absence of Center 2 may be due to the required presence of the Fp for one of its ligands. The [3Fe-4S] cluster is necessary for reconstituting purified Fp and Ip to the anchor domain of *B. taurus* SDH, suggesting its presence is necessary for assembly with the membrane domain (104).

I-4.d) The Anchor Subunits

SDH/FRD is anchored to the membrane by one (27 kDa) or two smaller (17 and 13 kDa) membrane proteins. The anchor domain attaches the catalytic dimer to the membrane, contains the quinone binding sites, and is often modified by a heme prosthetic group. The anchor domains of SDH/FRD enzymes have been classified into four structural groups (A-D) depending on the number of subunits and the presence or absence of heme prosthetic groups (46). Type A anchors have two subunits and two heme prosthetic groups. Type B anchors have one subunit and two heme prosthetic groups. Type C anchors have two subunits and one heme group. Type D anchors have two subunits but no heme. All four anchor types have been shown to share a common origin (46). Unlike the sequences of the catalytic subunits, the amino acid sequences of the anchor subunits are not well conserved between organisms. Their membrane topologies, however, are well conserved. In enzymes with two anchor subunits, such as *E. coli* SDH, *E. coli* FRD, and yeast SDH, each anchor subunit has three transmembrane helices. Type B SDH/FRD anchor domains, containing only one subunit, such as *B. subtilis* SDH and *Wolinella succinogenes* FRD, contain five transmembrane helices.

SDH and FRD ultimately donate or accept electrons to and from quinone. Since quinones are two electron carriers and Fe-S clusters are one electron carriers, the SDH/FRD enzyme must be able to stabilize a semiquinone species. The stabilization of the semiquinone intermediate (QH•) allows SDH and FRD to reduce or oxidize quinone in successive one electron transfers.

Different organisms use different quinones as acceptors or donors. Generally, SDH reduces the high potential quinone, ubiquinone, while FRD oxidizes the low potential quinone, menaquinone (140). *In vitro, B. subtilis* SDH and *W. succinogenes* FRD show little quinone specificity suggesting the quinone binding sites of SDH and FRD are not quinone-species specific.

Accumulating evidence indicates that there are likely two quinone binding sites in SDH/FRD. A proximal site is located on the inner, negative side of the membrane and appears to interact both with the [3Fe-4S] cluster, and the *b*-type heme if present (46). This proximal site is sensitive to the inhibitors 2-thenoyltrifluoroacetone (TTFA) and carboxin in mammalian SDH (98). In contrast, FRD of *E. coli* and SDH of *B. subtilis* are insensitive TTFA and carboxin, but they are sensitive to 2-*n*-heptyl-4-hydroxyquinolone-N-oxide (HQNO) (30, 133). This compound appears to interact with a distal quinone binding site on the outer, positive side of the membrane. Extensive mutagenesis studies on *E. coli* FRD found specific amino acids involved in quinone binding that correspond to both the proposed distal and proximal sites (149).

Most SDH/FRD complexes studied contain one or two prosthetic type-*b* heme groups. In enzymes with two heme groups, one is a high redox potential heme, and the other is a low redox potential heme. The heme groups in SDH/FRD enzymes are coordinated by two histidine residue ligands. In *E. coli*, the histidine ligands are located in both anchor subunits (101). In the yeast SDH anchor subunits, there are histidine residues in the Sdh3p subunit, but not in the Sdh4p subunit, that are appropriately located to serve as heme ligands (105). *E. coli* FRD is the best studied SDH/FRD enzyme with a type D anchor (containing no heme). The yeast SDH was originally placed in this category (130), however, the presence of a type-b heme was recently reported (105).

The physiological role of the heme group in SDH/FRD is still controversial. It seems clear that heme has a role in the assembly and structure of the membrane anchor but it is not known whether the heme group is a component of the electron transfer pathway. The midpoint redox potential of some heme groups is too low to be succinate reducible (157). *B. taurus* SDH has such a heme, but in experiments with enzyme preparations with a low heme content, ubisemiquinone species are not detectable (155). In addition, there are SDH/FRD enzymes with no heme at all such as the well studied *E. coli* FRD. Experiments on the kinetics of heme reduction in *W. succinogens* FRD demonstrated that electron transfer is at least as fast as the enzyme turnover, supporting the possibility that heme could be involved in electron transfer (46).

Heme has been demonstrated to be required for proper assembly of the holoenzyme. The absence of heme in *B. subtilis* SDH heme auxotroph mutants prevents the association of the soluble Fp and Ip SDH dimer with the membrane domain (35). Instead, the soluble subunits accumulate in the cytoplasm until heme is added (55). This suggests that heme is inserted into the anchor subunits after they have been targeted and assembled in the membrane (35).

In vitro, reconstitution of the catalytic domain to the anchor domain is sensitive to the ionic strength and the pH of the medium, indicating an electrostatic attraction between the anchor and soluble domains (2). In enzymes with two anchor subunits, both proteins are required for binding the catalytic dimer (27).

I-5. Assembly of SDH and FRD

Assembly of the electron transport chain complexes is a very complex process and there is much interest in elucidating the temporal steps involved. Multiple subunits must be synthesized, targeted to the correct location, properly processed to include any required cofactors, folded, associated with each other, and inserted into the membrane with the correct orientation. The process in eucaryotes is additionally complicated in that numerous nuclear-encoded proteins of the electron transfer complexes are synthesized in the cytosol, and must be properly targeted and imported into the mitochondrial matrix for processing and assembly.

SDH presents a good model for studying the assembly of mitochondrial enzymes as it is the simplest of the membrane-bound respiration enzymes eucaryotes with only four subunits, yet it still provides a sophisticated model because it contains up to five different prosthetic groups: a covalently bound FAD, three non-identical iron-sulfur clusters, and in some cases a *b*-type heme.

I-5.a) Order of Subunit Assembly

The overall order of subunit assembly and prosthetic group insertion is not entirely clear. Evidence suggests that the first step in assembling SDH and FRD begins with the insertion of the anchoring subunits into the membrane. Then, once the prosthetic groups have been attached to the appropriate subunits, the remaining subunits are able to associate with each other.

Time-course experiments of *E. coli* FRD assembly show that the rapid accumulation of the FRD anchor subunits (FrdC and FrdD) in the membrane is the initial step in FRD assembly (84). The assembly of the anchor subunits is not dependent on the presence of the Fp and Ip catalytic proteins, which is also consistent with a model of SDH assembly initiating with the insertion of the anchor domain (55). *B. subtilis* SDH mutants lacking the Ip or Fp subunits were still able to accumulate targeted anchor subunits (55).

The prosthetic heme group is likely inserted into the anchor domain after the subunits have been targeted to the membrane, based on the observation that the anchor subunit of *B. subtilis* is found present in the membrane without heme in heme auxotrophs starved of heme (35). In these mutants, the lack of heme prevents the binding of the catalytic proteins to the membrane, demonstrating the requirement for heme for proper holoenzyme assembly. The soluble subunits accumulate in the cytoplasm until exogenous heme is added and assembly can proceed. Thus, the heme group has an obligatory role in assembly.

The order of assembly of the Fp and Ip to the membrane domain is not clear. Evidence from various sources of SDH and FRD does not present a consistent model. In some instances, it appears that the Ip binds to the anchor domain followed by the association of the Fp, whereas in other cases, the soluble proteins appear to form a heterodimer prior to association with the membrane. The latter case is supported by observations of *E. coli* FRD and yeast SDH assembly. In the absence of anchor proteins, the catalytic heterodimer accumulates in the soluble cellular fraction as an active enzyme (2, 20, 86). In contrast, time-course studies of *E. coli* FRD assembly showed Ip binding to the membrane before associating with the Fp (84). When the soluble subunits are prevented from binding to the membrane, as with the *B. subtilis* SDH, the Ip and Fp accumulated in the soluble fraction but were not detected as a heterodimer (55). While the sequence of Fp and Ip binding to the membrane domain is unclear, it seems likely that the Ip and Fp are modified to contain their respective prosthetic groups prior to assembly with the membrane.

I-5.b) Insertion of Cofactors

The insertion of the Fe-S clusters in the Ip probably occurs before the Ip and Fp associate with each other. The [2Fe-2S] and [3Fe-4S] clusters are detected in the *E. coli* FRD Ip when the subunit is expressed in the absence of the Fp (69). The [4Fe-4S] cluster may provide a structural bridge between the subunits, and thus may only exist when the soluble subunits are associated. No definitive experiments have been able to determine whether or not Fe-S insertion is required for the assembly of the soluble subunits to the anchor subunits, however, reconstitution of *B. taurus* SDH is only possible when the [3Fe-3S] center is intact (104).

The binding of the FAD to the Fp is not required for assembly of the holoenzyme. The covalent attachment of the flavin to the Fp of the yeast SDH and the*E. coli* FRD was prevented by creating histidine to serine site directed mutants (9, 110). These mutants were still able to assemble properly, but only retained fumarate reductase activity. In addition, *B. subtilis* SDH Fp mutants have been isolated that are completely unable to bind FAD, but are still assembled into a holoenzyme (53). In yeast, flavinylation occurs in the mitochondrial matrix after the presequence has been removed, and the protein has obtained a sufficiently folded conformation (111). The presence of the Ip subunit stimulates FAD attachment (111). The non-covalent FAD yeast mutant is less stable then the wildtype enzyme (110). The covalent attachment of FAD to the Fp may make the protein less sensitive to degradation. In agreement, the covalent attachment of FAD to 6-HDNO also is reported to make the enzyme less susceptible to degradation (13). The covalent attachment may function to raise the midpoint potential of the FAD or contribute to structural stability. A C-terminal truncation of the yeast Fp was not flavinylated, suggesting that flavinylation is not a co-translation event and that the reaction requires a suitably folded conformation to proceed. Although the Ip subunit stimulates flavinylation of yeast Fp, it is not essential for attachment. In *B. subtilis* mutants which prevent binding of the soluble subunits to the membrane anchoring subunits, the Ip and Fp accumulate in the cytoplasm. The Fp is detected with a covalently bound FAD, but is not associated with the Ip (55). In agreement with these findings, *E. coli* FRD Fp expressed in the absence of the other subunits has covalently attached FAD (26). These lines of evidence suggest flavinylation in yeast occurs following import, proteolytic cleavage of the leader sequence, and after partial folding of the Fp, but before association with the Ip (111).

I-5.c) Proteolytic Processing During Assembly

Assembly of a multiple subunit complex is an intricate process. A number of additional accessory proteins and non-protein components are involved in assisting the various native polypeptides to reach their assembled, biologically active states at their final destinations.

Most eucaryotic SDH subunits are nuclear encoded. The individual subunits are translated in the cytoplasm and imported into the matrix of the mitochondria. The proteins are targeted to the mitochondria as precursor proteins containing N-terminal leader sequences. Mitochondria targeting N-terminal presequences generally are amphipathic alpha helices containing both positively charged and hydrophobic amino acids (143). Once imported into the matrix of the mitochondria, the precursors are converted into mature proteins by the proteolytic removal of the N-terminal sequence by the matrix processing proteinase (MPP) (51, 68). Yeast Fp belongs to a group of proteins that is converted into the mature form in two steps. The matrix processing proteinase cleaves off a fragment of the leader sequence creating an intermediate form of the protein. Proteolytic processing is completed by the mitochondrial intermediate proteinase (MIP) that cleaves the remaining eight amino acids (72).

I-5.d) Accessory Folding Proteins

A number of accessory proteins are involved in assisting native polypeptides to reach their biologically active states. An important group is chaperones, which function to maintain proteins in appropriate conformations to enable processes such as transport or folding. Molecular chaperones prevent polypeptide chains from forming incorrect associations with itself or other proteins (57). They do not become components of the final polypeptide structure and they are usually not substrate specific. Chaperones bind to a large number of polypeptides and are conserved throughout eucaryotic and procaryotic organisms. This group of proteins is also significant in roles other than correct folding. They are involved in intracellular transport, dissolution of protein aggregation and limiting the damage caused by stress, such as heat shock (89, 106). Chaperones promote correct folding in one of two ways: 1) they shield the hydrophobic surfaces to prevent aggregation or, 2) they isolate the protein from the surrounding cellular environment allowing folding to proceed until a stable state is reached. Hsp70 and Hsp60 represent two basic classes of chaperones, which are responsible for performing these roles. The 70-kDa heat shock protein (Hsp70), binds to peptides that are in a highly unfolded state. The *E. coli* homolog is the DnaK chaperone. Hsp70 binds proteins that are being translated or translocated across membranes (21). Two smaller accessory proteins, DnaJ and GrpE, modulate the activity of the Hsp70 class of chaperones. DnaJ bound in conjunction with Hsp70 stimulates Hsp70 ATPase activity, and GrpE homologs are nucleotide exchange factors (88). Hsp70 requires ATP hydrolysis to release its proteins (34).

The second important class of chaperones is composed of the 60-kDa heat shock proteins (Hsp60), sometimes referred to as chaperonins. The bacterial equivalent is the GroEL chaperone complex. These chaperonins are found in procaryotic cells and in eukaryotic organelles such as mitochondria (32). Proteins bound by GroEL possess some secondary structure and are predicted to be in an intermediate 'molten globule' state (45, 94). Hsp60/GroEL forms a large central cavity where the polypeptides bind folding intermediates (45, 82). This protective cavity isolates them from the surrounding environment and allows folding to occur without incorrect associations that might lead to aggregation (50). GroEL functions in conjuntion with GroES. The mitochondrial equivalent of GroES is Hsp10 (92). Hydrolysis of ATP is required for the release of bound proteins which involves interaction with GroES (39, 94). Release is thought to occur when the protein reaches a conformational state in which the chaperone no longer recognizes it as a substrate (7). Assembly of the SDH/FRD enzyme complexes involves many steps and the proper conformation must be maintained. It is possible that chaperones play a crucial role in this process.

I-6. Genes Encoding SDH and FRD

The genes for the SDH and FRD complexes have been cloned and sequenced from numerous procaryotic and eucaryotic organisms. The sequences encoding the individual Fp and Ip subunits are highly conserved. The sequences for the anchor subunits appear to have diverged greatly, but the overall structural morphology has been conserved.

I-6.a) Eucaryotic SDH/FRD Genes

In eucaryotes, the individual subunits are encoded by unlinked nuclear genes, which encode precursor proteins. The genes are translated in the cytoplasm with an N-terminal leader sequence. The leader sequence directs the proper targeting of the subunits to the mitochondria. Recent reports have identified a few eucaryotes that contain the genes for various SDH subunits within their mitochondrial genome instead of their nuclear genome. The mitochondrial genomes of the photosynthetic red algae *Chondrus crispus*, *Porphyra purpurea*, and *Cyanidum caldarium* as well as the mitochondrial genomes of the zooflagelate *Rectinomonas americana* and the liverworth *Marchantia polymorpha* carry the genes for SdhC and SdhD subunits (19, 85, 141). *Porphyra purpurea* also contains the gene for the Ip subunit in the mitochondrial genome. In the mitochondrial genomes of *P. purpurea* and *R. americana*, the *sdhC* and *sdhD* genes are arranged in a similar fashion as seen in bacteria, while in *C. crispus* and *M. polymorpha* the genes are separated from each other. These cases are most likely exceptions in which the transfer of the mitochondrial genes to the nucleus has not yet occurred (127).

In many animals, a complex corresponding to FRD has not been detected. However, some SDH enzymes such as the *Ascaris suum* (75) and *Desulfobulbus elongatus* (122) SDH, have been suggested to function as both an SDH and FRD *in vivo*. The parasitic nematode, *A. suum*, belongs to the group of eucaryotic organisms including protozoa, parasitic helminths, and some marine annelids and mussels, that appear to have multiple genes for individual subunits of Complex II whose expression is related to the life cycle (140). *Ascaris suum* has two different stage-specific forms of Fp expressed differentially in the adult anaerobic parasitic stage, and the aerobic free-living larval stage (125). The two Fp polypeptides are distinguishable by peptide mapping and electrophoresis experiments (125). There is also a report of a sheep nematode, *Haemonchus contortus*, that expresses two stage-specific forms of the Ip subunit during the aerobic and anaerobic life cycles (116). To date, not all the genes for the subunits of these complexes have been isolated, so it is not known if these life-cycle dependent complexes differ for all four subunits, or in just one or two. However, the use of alternative stage-specific subunit isozymes has been proposed to be responsible for enabling the switch in energy metabolism from an aerobic larval metabolism to an anaerobic adult metabolism by allowing the SDH complex to function alternatively as a conventional mitochondrial SDH at one stage of development and as a FRD enzyme during a different stage. An additional consideration in the ability to alter the direction of electron flow through the SDH complex, is the quinone species that is employed. *A. suum* SDH was demonstrated to switch from the use of ubiquinone (E_m =+112 mV) under aerobic conditions, to rhodoquinone (E_m =-63 mV) under anaerobic conditions (140). *E. coli* is known to express either SDH or FRD depending on the environmental condition (60) and produces ubiquinone aerobically and menaquinone anaerobically (146). However, when overexpressed in sufficient quantities, these enzymes can functionally replace each other *in vivo* demonstrating their abilities to utilize both quinone species (27, 93).

In yeast, the genes encoding the SDH Fp, Ip, and anchor subunits are named *SDH1, SDH2, SDH3,* and *SDH4* respectively in order of decreasing size. The genes code for precursor proteins that contain N-terminal leader sequences, which are cleaved following import into the mitochondrial matrix. All of the genes have been cloned and sequenced (18, 28, 90, 113).

I-6.b) Procaryotic SDH/FRD Genes

In procaryotes, the SDH and FRD genes are encoded as operons. All four genes encoding the Fp, Ip, and anchor subunits are under the control of a single promoter. Under inducing conditions, they are cotranscribed into a single polycistronic mRNA molecule. In facultative bacteria, such as *E. coli*, there are two separate operons, each encoding either the SDH complex or the FRD complex (60). In bacteria, the genes for the Fp, Ip, and anchor subunits of the SDH and FRD complexes are named *sdhA/frdA, sdhB/frdB, sdhC/frdC,* and *sdhD/frdD,* respectively. The *E. coli sdh* operon maps at 17 min on the chromosome and is arranged in the order *sdhCDAB* (29, 154). The same gene order is found in the *sdh* operons of the bacteria *Paracoccus denitrificans* and *Coxiella burnettii* (56). In *B. subtilis, Bacillus macerans* and *Wollina succinogenes,* which all contain a Type B anchor (one anchor subunit) the order is *sdhCAB* and *frdCAB* (77). The *E. coli frd* operon is arranged as *frdABCD*. This pattern of organization is also seen in *Haemophilus influensae, Proteus vulgaris,* and *Mycobacterium tuberculosis* (24). While the order of the genes varies depending on the species, the Fp gene is always immediately upstream of the Ip gene, and the anchor subunits are always arranged as SdhC/FrdC, SdhD/FrdD.

The *E. coli frd* operon has been well characterized. It is mapped at 94 min on the chromosome at a distant site from the *sdh* operon (81). It was originally cloned as a 4.9-kb *Hind*III fragment that was capable of conferring a Frd+ phenotype to a Frd- mutant. *E. coli* Frd- mutants are unable to grow anaerobically on a non-fermentable carbon source such as glycerol using fumarate as a terminal electron acceptor. The *frd* promoter is located 93 nucleotides upstream from the *frdA* coding sequence (70). Initiation of transcription produces a 3,450 nucleotide mRNA (70). The majority of *frdABCD* mRNA molecules terminate in a uridine-rich region 46 nt downstream of *frdD* following a proposed stem and loop terminator structure (70). A second minor terminator site is situated approximately 100 nt further downstream. Upstream of the *frd* genes, on the opposite strand is another coding sequence for a functionally unrelated gene (25). Downstream of the fourth gene, *frdD*, is the *ampC* gene, which encodes the chromosomal β-lactamase (67). The promoter for the *ampC* gene is contained within the coding sequence for *frdD*.

The *E. coli frd* operon has as number of unusual conserved genetic features (26). First, *frdA* employs the uncommon GTG initiation codon rather then the usual ATG initiation codon. Second, the operon displays extreme sequence economy. In fact, the coding regions of the first two genes, *frdA* and *frdB*, overlap by 8 nucleotides. The ribosomal binding site for *frdB* is contained within the coding region for *frdA*. In addition, the intergenic regions between *frdB* and *frdC*, and between *frdC* and *frdD* are atypically short. The significance of these molecular organizational features for FRD expression is not known.

I-6.c) Regulation of SDH and FRD Gene Expression

Facultative organisms are able to proliferate under both aerobic and anaerobic conditions. This is possible because a variety of electron transfer chains are present. Generally, the chains consist of a primary dehydrogenase that passes reducing equivalents on to a terminal electron acceptor via a membrane bound quinone pool. Depending on the substrates available, facultative organisms adapt their energy metabolism to utilize the most energetically favorable respiratory pathway by employing the electron donor-acceptor couple that yields the most energy. This adaptation takes place rapidly and involves the tight regulation and coordination of the expression of the alternative respiratory pathway genes.

The expression of FRD and SDH genes is tightly regulated based on the environmental conditions. Generally, SDH is expressed maximally under aerobic conditions when succinate is present as the main source of carbon and energy and is repressed under anaerobic conditions. FRD is induced under anaerobic conditions on non-fermentable carbon sources and is maximally expressed when fumarate is present and nitrate is absent.

The single-celled eucaryotic organism, yeast, is a metabolically versatile facultative anaerobe that has the capacity to grow on a wide variety of carbon and nitrogen sources. The carbon source utilized can be either a fermentable or nonfermentable substrate. Growth on a non-fermentable source requires the presence of oxygen, while fermentable substrates may be metabolized anaerobically by deriving ATP solely from glycolysis. Yeast expresses different sets of metabolic pathway enzymes under different growth conditions. The expression of yeast SDH depends on oxygen availability and the carbon source. The presence of an abundance of glucose causes the catabolite repression of a large number of mitochondrial yeast proteins including SDH. Yeast that are grown on a glucose-rich medium have mitochondria with very poorly defined inner membrane cristae as a result of the repression of the biogenesis of the inner membrane protein complexes. Regulation of the yeast SDH has been studied by investigations of *SDH2* (Ip) expression. These studies have shown that glucose results in both a repression of transcription and a reduction in the half-life of the *SDH2* mRNA (91).

E. coli is a facultative bacterium that can grow on a number of substrates during aerobic and anaerobic growth (64). The terminal electron acceptors utilized by *E. coli* in decreasing order of favorable energy yield are oxygen (+800 mV), nitrate (+420 mV), dimethyl sulfoxide (DMSO: +160 mV), trimethylamine N-oxide (TMAO: +130 mV), and fumarate (+30 mV) (26). The bacteria selectively expresses the enzymes specific to the pathway whose electron donor-acceptor couple yields the most energy (43). There are three global bacterial regulatory systems that provide transcriptional control to coordinate respiratory enzyme synthesis in response to anaerobiosis and nitrate. The two systems that respond to variable oxygen levels are the ArcA/ArcB and the FNR systems. Generally, the ArcA/ArcB global regulatory system is responsible for the repression of genes involved in aerobic metabolism, while FNR is activated under anaerobic conditions and functions as both a transcriptional activator and repressor of genes involved in anaerobic and aerobic metabolism. The NarX/NarL system responds to nitrate levels.

The ArcA/ArcB and NarX/NarL systems belong to a two-component family of regulatory systems, where one component detects an environmental signal and then modifies the activity of the second component. Generally the ‘sensor’ protein is a membrane bound kinase, while the ‘reponse-transmitter’ component is a cytoplasmic protein containing a DNA-binding domain.

FNR has significant amino acid sequence similarity to the regulator DNA-binding cyclic AMP receptor protein (CRP) that mediates catabolite repression in *E. coli* (132).

However, FNR has an N-terminal cysteine-rich extension that ligates an iron-sulfur redox group. The mechanism of oxygen sensing by the ArcA/ArcB and the FNR sensors are not clear, however they are thought to be distinct. The iron-sulfur redox group of FNR may be responsible for sensing anaerobiosis and cause a conformational change or dimerization of FNR under anaerobic inducing conditions resulting in an active form of FNR that can then bind the regulatory elements of targeted promoters (95, 139).

Regulation of *E. coli sdh* expression is controlled by both the availability of oxygen and the source of carbon (Figure I-4) (118). Anaerobic repression of the *E. coli sdhCDAB* operon is mediated by the *arcA* and *arcB* gene products (66). The ArcA membrane protein serves as an oxygen sensor and under low oxygen conditions, it modifies ArcB to produce an active DNA binding species. Once activated, it binds in the vicinity of the *sdhCDAB* promoter and inhibits transcription. Glucose, as a carbon source, leads to the catabolite repression of *E. coli* SDH expression via cAMP and the regulatory DNA-binding CRP protein.

The expression of *E. coli frd* varies depending on the presence of nitrate and anaerobiosis. FRD is induced under anaerobic conditions when fumarate is present and the preferred electron acceptor, nitrate is unavailable. During anaerobic induction, nitrate results in a 25-fold repression of *frd* expression (71). Fumarate induces expression 2-3 fold in the presence or absence of oxygen (71). The anaerobic induction of the *E. coli frd* operon is mediated by the positive-regulatory protein, FNR, which is activated under anaerobic conditions and binds to the *frd* promoter to activate transcription (81). The nitrate repression of *E. coli frd* is mediated by the NarX and NarL regulatory proteins (103). NarX is a membrane associated sensory protein that detects nitrate and modifies the regulatory protein NarL (135). Once activated, NarL becomes able to bind DNA and represses the expression of the *frd* operon.

I-7. Thesis Objective

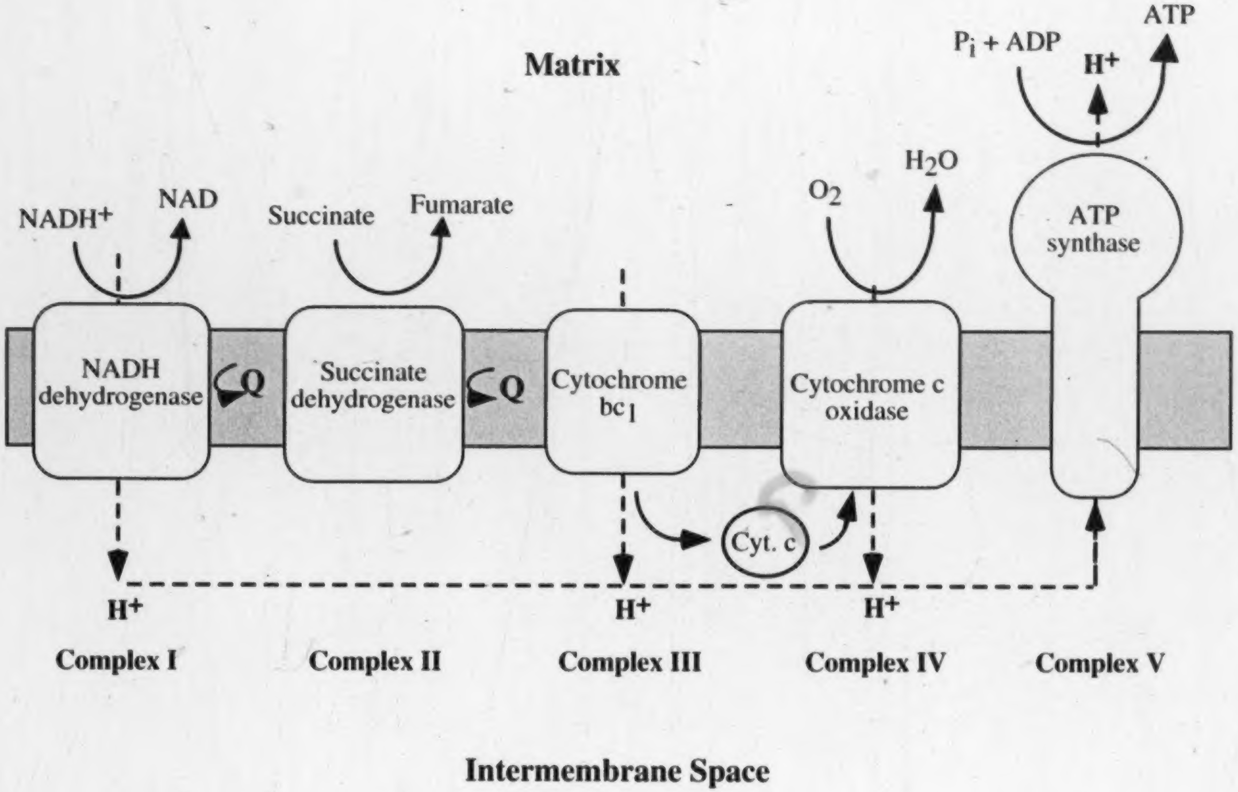
The work in the Lemire laboratory is focused on studying the biogenesis, structure, and function of the yeast mitochondrial SDH complex. This model system would be improved if it were possible to purify functional yeast SDH in sufficient quantities for mutational and biophysical studies. The aim of this thesis is to design and create a yeast SDH expression system in *E. coli*. The design and rationale is based on the *E. coli frd* operon. The *E. coli* FRD complex has been previously successfully overproduced 30-40 fold by cloning the *E. coli frd* operon into a multicopy plasmid and inducing expression under anaerobic conditions (86).

We have designed a synthetic yeast SDH operon encoding all four mature yeast subunits under the control of the *E. coli frd* promoter. The synthetic yeast SDH operon was tested for expression in the *E. coli* strain, HB101 grown anaerobically on a non-fermentable carbon source. Identification of the yeast polypeptides produced was performed using subunit-specific antisera against yeast Sdh1p, Sdh2p, and Sdh3p following separation by SDS-PAGE. No antiserum against the yeast Sdh4p is available. Results indicated that expression under the *frd* promoter was either low, or produced unstable polypeptides. Antisera against Sdh2p and Sdh3p detected a low accumulation of immunoreactive protein during a brief window of time about 12 hr after induction of expression.

The synthetic yeast SDH operon was also placed under the control of the T7 bacteriophage promoter to test expression and stability over a period of minutes. Expression was induced by heat-shock under aerobic growth conditions. The yeast polypeptides produced were detected by either radiolabeling or immunodetection. The T7 expression system successfully identified polypeptides corresponding to Sdh1p, Sdh2p, and Sdh3p.

The expression of the yeast SDH operon failed to produce a high enough level of enzyme for use in further studies. Several approaches to improving the level of expression of the synthetic yeast SDH operon are outlined in the future studies section in the discussion chapter.

Figure I-1. Schematic Representation of the Mitochondrial Transport Chain



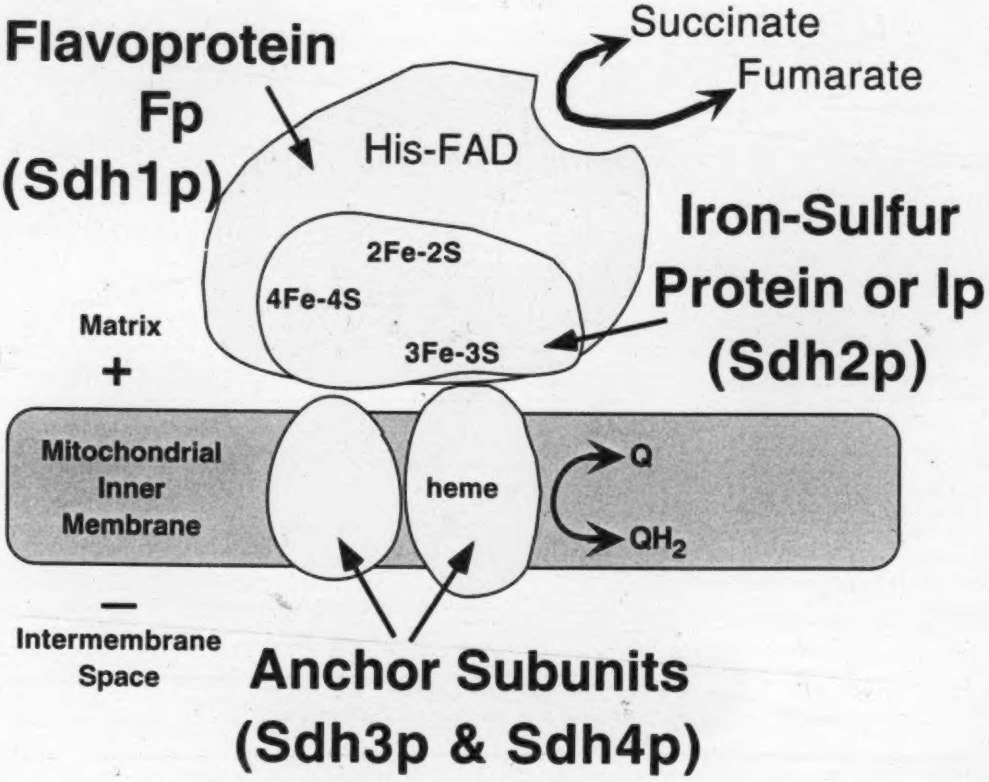


Figure I-2. Schematic of the *Saccharomyces cerevisiae* Succinate Dehydrogenase Enzyme

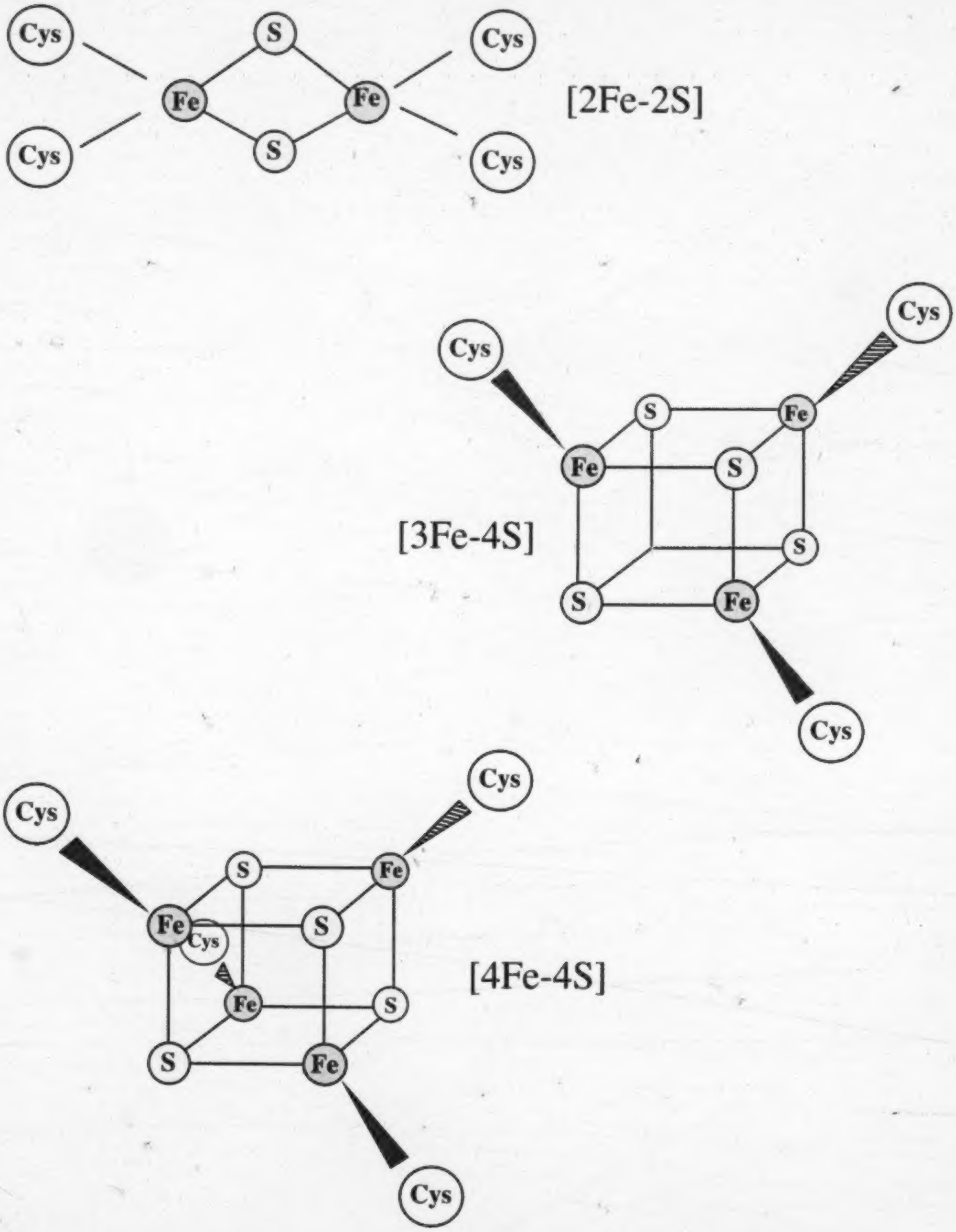


Figure I-3. Molecular Structure of [2Fe-2S], [3Fe-4S], and [4Fe-4S] Clusters.

The ligand sulfur atoms of the cysteine amino acids are shown as Cys. The inorganic sulfur atoms are shown as S. The iron atoms are shown as Fe.

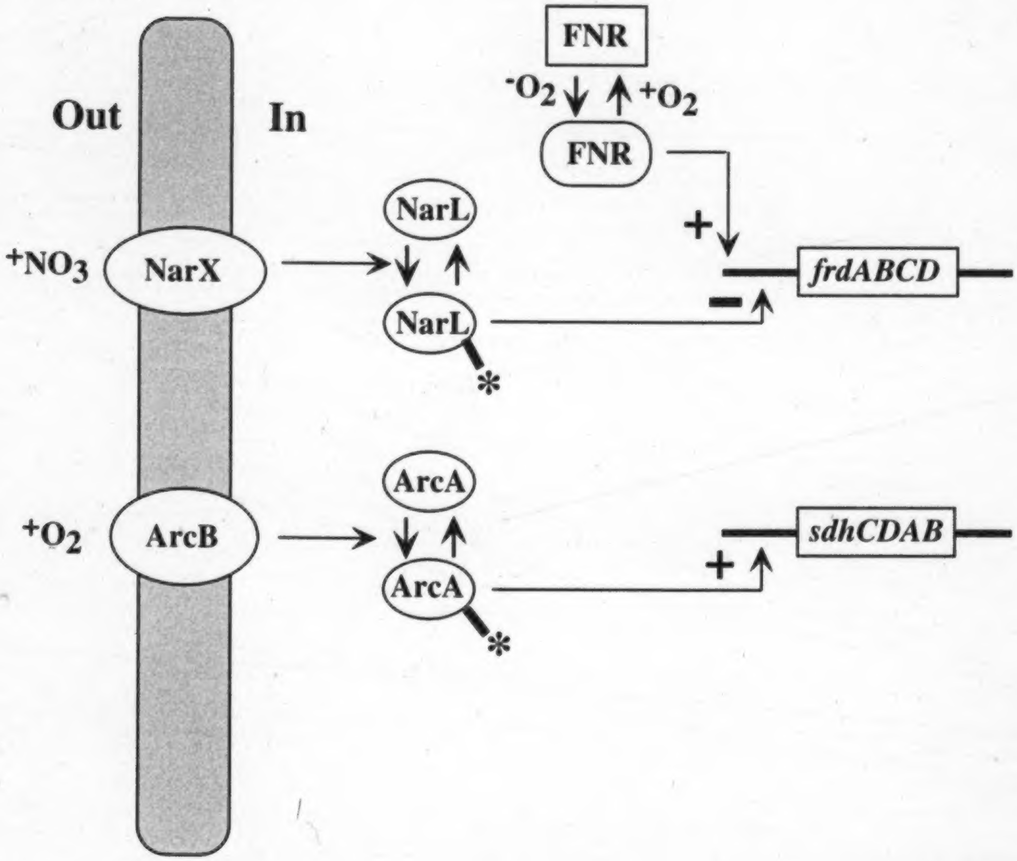


Figure I-4. Regulatory Scheme for Oxygen and Nitrate Control of *E. coli sdh* and *frd* operon expression.

The three global regulators ArcA/ArcB, NarX/NarL, and FNR provide transcriptional control to coordinate the synthesis of various metabolic pathway enzyme complexes in response to environmental signals. The model shows the stimuli (O_2, nitrate), the sensors (ArcB, NarX) the response regulators (ArcA, NarL), and the sensory-regulator FNR modifying the expression of the *E. coli sdh* and *frd* complexes. Transcriptional activation (+) and repression (-) of the operons is shown.

Chapter II: MATERIALS AND METHODS

II-1. Strains and Plasmids

The *Escherichia coli* and *Saccharomyces cerevisiae* strain genotypes are listed in Table II-1. *E. coli* strain DH5α was used routinely for plasmid propagation and isolation during cloning. GM48 was used when the isolated plasmid DNA was to be digested with the methylation sensistive restriction enzyme *Bcl* I. INVαF′ was used during TA cloning. HB101 was used for anaerobic, *frd* promoter expression studies. K38/pGP1-2 was used for T7 promoter/T7 RNA polymerase ^{35}S-methionine labelling and expression studies. Three yeast strains, D273-10B, Sdh1Ad1, and Sdh3W3, were used for preparing mitochondria to be used as antibody controls on immunoblots.

A list of plasmids and vectors used is shown in Table II-2. The plasmids and vectors used in the construction of the recombinant expression vectors are as follows. pBC KS$^-$, pBluescript II SK$^{+/-}$, and pUC19 were used as cloning vectors during plasmid construction. pCR2.1 is a vector from Invitrogen that is designed for cloning PCR products. Plasmids pRA, pBSII-SDHB, pGM205, pSDH4-17, and pfrd79 contain the cloned yeast genes *SDH1*, *SDH2*, *SDH3*, *SDH4*, and the *E. coli frd* operon, respectively. These were used as the source of wildtype genes for PCR templates during the construction of the synthetic yeast SDH operon. pKIXX contains a kanamycin resistance cassette gene. pGP1-2 was used during T7 promoter/T7 RNA polymerase expression studies. This plasmid, as developed by S. Tabor, contains a thermoinducible T7 RNA polymerase gene. The remaining plasmids in Table II-2 were constructed in this study.

II-2. Growth Media

For amplification and preparation of plasmid DNA, *E. coli* cells were routinely grown and subcultured in Luria broth (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl; (123)). Plasmids were maintained by the addition of antibiotics (ampicillin, 100 μg/mL; kanamycin, 40 μg/mL; chloramphenicol, 42.5 μg/mL) to the medium.

For anaerobic expression, precultures were prepared by aerobic growth overnight in LB containing antibiotics at 37 °C. Anaerobic induction was on a glycerol-fumarate medium (GF; per liter: 5.44 g of KH_2PO_4, 10.49 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, 3.75 g of NaOH, 4.64 g of fumaric acid, and 0.01% metal mix [0.4 mM $MgSO_4$, 25 μM $MnSO_4{\bullet}H_2O$, 10 μM $FeSO_4{\bullet}7H_2O$, 5 μM $CaCl_2$], 0.75% (v/v) glycerol, 0.05% (w/v) casamino acids). The medium was supplemented with 20 μg/mL each of thiamine, proline, and leucine. Anaerobic growth was at 37 °C in 250 mL centrifuge bottles filled to the top with GF medium (for a total volume of 290 mL), sealed air-tight with a rubber-seal lined screw cap. Sedimentation of cells during anaerobic growth was prevented by gentle stirring.

For bacteriophage T7 promoter/T7 RNA polymerase expression studies, cells were grown in LB. During labeling with ^{35}S-methionine/cysteine, M9 minimal medium supplemented with Difco Methionine Assay Medium (0.2%), thiamine (20 μg/mL) and glucose (1%) was used.

II-3. General DNA Isolation, Purification, and Cloning Techniques

Unless otherwise stated, methods employed are essentially as described in Sambrook *et al.*, 1989 (123). Plasmid DNA was routinely isolated for cloning and restriction analysis by a modified alkaline-lysis method (97). Further purification of both plasmid DNA and polymerase chain reaction-generated DNA was accomplished using glass milk powder (142). Double-stranded plasmid DNA used for sequencing was prepared by either the Modified Alkali-Lysis/PEG Precipitation Procedure (4) or Qiagen Plasmid Mini Preparation utilizing the QIAGEN-tip 20 kit. Routinely, ethanol precipitaion of DNA was carried out using the selective alcohol precipitation protocol (65) by adding 1/2 volume 7.5 M NH_4CH_3COO, mixing, and then adding 9 volumes of ice-cold 95% ethanol, and precipitating at -70°C for at least 15 min.

Restriction endonuclease enzymes were purchased from New England Biolabs (NEB), Gibco BRL Life Technologies (BRL), Promega, or Boehringer Mannheim (BM). Enzyme digestions were carried out for 1-2 hr using conditions specified by the manufacturer. Reactions were stopped by the addition of 0.1 vol of 10x stop mix (50% glycerol, 0.5% bromophenol blue, 0.5 mM EDTA). DNA was analysed by gel electrophoresis in 1% agarose gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Restriction fragment sizes were determined relative to molecular weight standards run in adjacent lanes. For isolation and gel-purification of restriction fragments for cloning, DNA was electrophoresed in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), excised as a gel-slice, and recovered using glass milk powder (142).

Ligations were carried out in a final volume of 10 μl using up to 1 μg of DNA, and 1 Unit T4 DNA Ligase (New England Biolabs). Routinely, vector:insert ratios of 1:1, 1:3, and 1:0 (negative control) were used. For transformation into *E. coli*, 1 μl of the ligation reaction was used.

Transformation of *E. coli* was carried out by electroporation (48) using the Gibco BRL Cell-Porator *E. coli* Electroporation System at 2.44 kV. For selection on kanamycin or chloramphenicol plates, the transformants were allowed to recover in 1 mL LB without antibiotics for 1 hr at 37 °C before plating on selective LB plates. Correct transformants were confirmed by restriction analysis following plasmid isolation from single colonies.

II-4. Di-/Trinucleotide Sticky End Cloning (Di/Trisec)

PCR products were routinely cloned using Di-/Trinucleotide Sticky End Cloning (Di/Trisec) as developed by Dietmaier and Fabry (10). This method relies on the generation of complimentary di- or trinucleotide sticky ends on linearized plasmid vector and PCR-amplified insert DNA. For this technique all PCR primers were designed to contain a 5′-ATCG-3′ sequence on the 5′ end of the primers to allow for cloning into a *Bam*H I site following enzymatic modifications. Briefly, the 4-bp 5′ overhang ends on the vector, produced by digestion with *Bam*H 1 (GATC), are converted to 3-bp ends (GAT) by partially filling in with Klenow enzyme in the presence of dGTP. The PCR fragment ends (ATCG) are converted to 3-bp 5′ overhang complimentary ends (ATC) by trimming with controlled 3′-5′ exonuclease activity of T4 DNA polymerase in the presence of a "stopping nucleotide" (dCTP).

Specifically, vector DNA for Di/Trisec cloning was prepared in the following manner: vector DNA was digested with *Bam*H 1 for 2 hr. Next, dGTP (final concentration 0.1 mM) and 4 Units Klenow enzyme were added directly to the digestion reaction and incubated at room temperature for 15 minutes. The reaction was stopped by heating to 75 °C for 15 min. The DNA was purified by ethanol precipitation and resuspended in dH_2O for use in ligation.

Each PCR-generated insert was prepared for Di/Trisec cloning as follows. On ice, in the following order; 2µl of PCR reaction, 1.5 µl 2 mg/mL BSA, 7 µl 5x T4 DNA polymerase buffer (165 mM Tris-acetate, pH 7.9, 330 mM sodium acetate, 50 mM magnesium acetate, 500 µg/mL BSA, 2.5 mM DTT), 1.5 µl 2 mM dCTP, 20 µl sterile water and 3 µl 1 U/µl T4 DNA Polymerase were mixed and subsequently incubated at 12 °C for 30 min. Reactions were stopped by heating to 80 °C for 15 minutes. The trimmed DNA inserts were purified by glassmilk powder and ligated to prepared vector DNA overnight at 15 °C.

II-5. Polymerase Chain Reaction

PCR was performed using the Expand™ Long Template PCR System kit from Boehringer Mannhein following the manufacturer's general instructions. The kit contains a unique enzyme mix composed of the thermostable *Taq* and *Pwo* DNA polymerases designed for providing both a high yield and high fidelity amplification.

All reactions were carried out in a final volume of 50 µl using thin-walled PCR reaction tubes. For each reaction, two separate master mixes were prepared on ice. Master Mix 1 consisted of 350 µM of each dNTP (dATP, dTTP, dCTP, dGTP), 300 nM of the upstream primer, 300 nM of the downstream primer, and 200-500 ng template plasmid

DNA. Master Mix 2 consisted of 1x Expand™ PCR Buffer 1 (50 mM Tris-HCl, pH 9.2, 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$) and 0.75 µl enzyme mix supplied by kit. The two Master Mixes were pipetted together on ice, and cycling was immediately initiated. In general, denaturation was for 30 sec at 94 °C, annealing for 30 sec at 45-55 °C, and elongation for between 2 and 3.5 min at 68 °C. The cycle was repeated 25 times.

The amplified products were ethanol precipitated and resuspended in sterile water.

II-6. Oligonucleotide Synthesis

The oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA Synthesizer by the DNA Core Facility in the Department of Biochemistry, University of Alberta.

II-7. Construction of the Synthetic Yeast SDH Operon

A schematic diagram outlining the construction of the synthetic yeast SDH operon is shown in Figure II-1. The SDH operon was based on the *E. coli frd* operon and was designed to express yeast SDH in *E. coli*. The 5.1-kb fragment of DNA consists of the *E. coli frd* promoter, the four genes for the mature yeast SDH subunits, and the *frd* operon 3′ terminator sequence (Figure 3-1). A detailed description of the design of the operon is presented in Chapter 3. The operon was constructed in four steps as described below.

II-7.a) Isolation of the Yeast SDH Genes

The first step of constructing the SDH operon consisted of isolating the four yeast SDH genes and the two upstream and downstream *frd operon* transcription control elements (Figure II-1). PCR was used to amplify the six fragments of the SDH operon using specifically designed primers. Twelve primers, named SDH01-SDH12, were synthesized to amplify the six fragments named I-VI, respectively.

The Frd sequences were amplified using template of wildtype *frd*ABCD operon from plasmid pfrd79. The modified yeast SDH genes were amplified using individually cloned genes (see Table II-3 for plasmids used as PCR template; see Table II-2 for a description of plasmids). PCR was performed as detailed in Section II-5: "Materials and methods: Polymerase Chain Reaction". The details of the individual PCR reactions are presented in Table II-3. Primer sequences are listed in Table II-4.

Fragment I, generated by primers SDH01 and SDH02, is expected to be 843-bp in length and constitute nucleotides 1-833 of the SDH operon. It is flanked by the introduced restriction sites *Bam*H I (1-6) and *Sac* II (828-833). This fragment is essentially the 5′ *frd* sequence. It includes the entire desired region of the 5′ *frd* sequence including the *frd* promoter as well as the first 45-bp of the gene encoding yeast *SDH1*. Thus primer SDH02 is responsible for creating the junction between the 5′ *frd* sequence and the open reading frame of the first gene encoded in the operon, *SDH1*.

Fragment II is expected to be 1802-bp in length and encodes nucleotides 828-2619 of the yeast SDH operon. Nucleotides 828-833 are the introduced *Sac* II restriction site where fragment I will be joined to fragment II. The 3′ restriction site of fragment II is *Bsi*W I (2614-2619). Fragment II is essentially the *SDH1* gene, but lacks both the N-terminus and the C-terminus.

Fragment III is 785-bp in length and encodes nucleotides 2614-3388 of the SDH operon. It is essentially the *SDH*2 gene. It extends from the *Bsi*W I restriction site (2614-2619) in *SDH1*, through the stop codon of *SDH1*, the SDH1-SDH2 intergenic region, and the entire *SDH2* gene ending at the introduced *Afl* II restriction site (3383-3388). Thus, primer SDH05 is responsible for creating the SDH1-SDH2 junction of the SDH operon.

Fragment IV is essentially the *SDH3* gene. Primers SDH07 and SDH08 are expected to produce a 494-bp fragment constituting nucleotides 3383-3864 of the SDH operon. The introduced restriction sites are 5′ *Afl* II (3383-3387) and 3′ *Bcl* I (3859-3864). The fragment includes the stop codon for *SDH2*, the SDH2-SDH3 intergenic region, the entire *SDH3* gene, the SDH3-SDH4 intergenic region, and the first codon of yeast Sdh4p. Thus primer SDH07 is responsible for creating the SDH2-SDH3 synthetic junction, and primer SDH08 is responsible for creating the SDH3-SDH4 junction.

Fragment V is essentially the *SDH4* gene. It is expected to be 470-bp in length and constitute nucleotides 3859-4318 of the SDH operon. Fragment V extends from the *Bcl* I restriction site (3859-3864) to the *Mlu* I restriction site (4313-4318).

Fragment VI is 786-bp in length and encodes for the *frd* operon 3′ terminator sequence. It extends from the *Mlu* I restriction site (4313-4318) to the *Bam*H I restriction site (5085-5090), and constitutes nucleotides 4313-5090 of the SDH operon. Primer SDH11 creates the *SDH4-frd* 3′ terminator junction, and primer SDH12 adds on the *Bam*H I restriction site (5085-5090).

II-7.b) Construction of Fragment Dimers

The second step in constructing the operon consisted of making three dimers from the PCR-amplified fragments I-VI (Figure II-1). The fragments were combined by using the restriction endonuclease sites introduced between the genes to combine the DNA fragments. Generally, 1 μg of each PCR-fragment was digested, purified by ethanol precipitation, and ligated. To prevent self ligation, one fragment of each ligation was treated with shrimp alkaline phosphatase (United States Biochemical).

Specifically, dimer 1 was created by digesting fragment I and fragment II with *Sac* II overnight, and ligating for 16 hr at 4 °C. Dimer 2 (Fragments III-IV) was created by digestion with *Afl* II for 4 hr followed by ligation overnight at 15 °C. Dimer 3 (fragments V-VI) was created by digestion with *Mlu* I for 6 hr and ligating overnight at 15 °C.

Correct dimers were selectively amplified by PCR from the ligation reactions using outside primers (Table II-5) in order to obtain enough material to clone. 1 µg of the 10 µl ligation reaction was used as template in each case. PCR was carried out as detailed in "Materials and Methods: Polymerase Chain Reaction". The primers used and the PCR conditions are listed in Table II-5. The amplified products were purified by glassmilk powder and resuspended in sterile dH_2O and analyzed on a 1% agarose gel.

II-7.c) Cloning the Gene Dimers

The third step in constructing the synthetic yeast SDH operon consisted of cloning the three dimers (Figure II-1).

Plasmids pBSd1, pBSd2, pBSd3 Plasmid pBSd1 containing dimer 1, pBSd2 containing dimer 2, and pBSd3 containing dimer 3 were constructed by cloning each dimer into the multicopy vector pBluescript SK^+. They were each cloned by the Di-/Trisec as detailed in "Materials and Methods". This method was possible because the twelve primers used in the construction of the operon all had the 4-bp 5′-ATCG-3′ sequences designed onto the 5′ends as required for cloning into a *Bam*H I site.

II-7.d) Construction of the Synthetic Yeast SDH Operon From Cloned Dimers

In the fourth step, the cloned dimers were used in two subcloning steps as outlined in Figure II-1, to obtain a plasmid containing the entire synthetic yeast SDH operon.

Plasmid pBSd23 The 1238-bp *Bcl* I/*Spe* I fragment of pBSd3 containing dimer 3 was cloned into the *Bcl* I/*Spe* I site of pBSd2 resulting in plasmid pBSd23 containing both dimers 2 and 3 in the proper orientations. Plasmids pBSd2 and pBSd3 encoding dimers 2 and 3, respectively were isolated from the methylation-deficient bacterial strain GM48 to allow for enzymatic digestion with *Bcl* I.

Plasmid pBS123 The operon was completed by cloning the 2483-bp *Bsi*W I/*Spe* I fragment from plasmid pBSd23 containing fragments III-VI into the *Bsi*W I/*Spe* I site of vector pBSd1 resulting in plasmid pBS123 containing the entire synthetic yeast SDH operon as a *Bam*H I cassette in pBluescript II SK (Figure II-1).

II-8. Modifications To Correct the Synthetic Yeast SDH Operon:

II-8.a) Replacement of an Internal NgoM I/EcoR I fragment in the SDHI gene

Two convenient unique restriction sites within the *SDHI* gene of fragment II of the SDH operon were used to replace a 1.5-kb PCR-generated region of the SDH operon with the genomic DNA contained on plasmid pRA in order to avoid DNA sequencing of this region. The steps for replacing the internal fragment, as described below, are shown in Figure II-2.

Plasmid pUC19e The multicopy vector, pUC19, was modified to remove the *Eco*R I restriction site. The vector was digested with *Eco*R I, and the sticky ends were filled in by incubation with 1 U Klenow enzyme in the presence of 0.5 mM dNTP. The vector was then ligated overnight.

Plasmid pUC19e123 The 5.1-kb *Bam*H I fragment of pBS123 containing the yeast SDH operon cassette was cloned into the *Bam*H I site of vector pUC19e. In pUC19e123, the *Eco*R I and *Ngo*M I sites are unique.

Plasmid pUC19e123:kan A kanamycin-resistance cassette from plasmid pKIXX was isolated as a *Sma* I fragment, gel purified, and cloned into the *Stu* I site of pUC19e123. The plasmid pUC19e123:kan contains the SDH operon with a disruption of the *SDH1* gene at the *Stu* I site. The *Ngo*M I/*Eco*R I fragment containing a kan^R cassette can be easily distinguished from the non-interrupted genomic *SDH1* *Ngo*M I/*Eco*R I fragment derived from the plasmid, pRA.

Plasmid p123R The 2.9-kb *Ngo*M I/*Eco*R I::kan fragment in pUC19e123:kan was replaced with the 1.5-kb *Ngo*M I/*Eco*R I fragment from the genomic *SDHI* clone from plasmid pRA. Desired restriction fragments were isolated, purified, and ligated. Correct clones were confirmed by the loss of kanamycin resistance, restriction fragment analysis, and PCR. The resulting plasmid, p123R, contains the SDH operon in pUC19e with a 1.5-kb internal fragment of the *SDH1* gene derived from a source of cloned genomic DNA.

II-8.b) Correction of Dimer 2 of the Synthetic Yeast SDH Operon

Dimer 2 required being replaced as there was a PCR-generated sequence error in the region joining the two genes. To correct the error, the fragments III and IV were first individually cloned and sequenced, and then cloned into the operon as follows (Figure II-3).

Plasmid pBCsdh3m The fragment IV produced by PCR was cloned into the *Bam*H I site of plasmid pBC KS⁻ using the Di-Trinucleotide Sticky-End Cloning method, creating pBCsdh3m. The clone was confirmed by sequencing in one direction.

Plasmid pTAsdh2m The fragment III was produced by PCR with the following modifications from Table II-3. The annealing temperature was raised to 49 °C, the extension temperature was changed to 72 °C, and *Taq* polymerase was used instead of the Expand ™ enzyme mix. The PCR-generated fragment III was cloned using the TA Cloning System™ of Invitrogen following the manufacturer's instructions. Immediately following amplification by PCR, the monomer was purified using glass powder and ligated overnight with the provided pCR2.1 vector. The ligation was transformed into INVαF′ competent bacterial cells by heat shock following the manufacturer's instructions, and selected for on LB +kan plates containing 32 µg/mL X-Gal. The clone was confirmed by sequencing in one direction.

pSdhOpF Plasmid pSdhOpF was constructed in a triple ligation containing p123R with an incorrect dimer 2 removed by a *Bsi*W I/*Bcl* I digestion as the vector, fragment III digested with *Bsi*W I and *Afl* II, and fragment IV digested with *Afl* II and *Bcl* I. This created plasmid pSdhOpF which has a corrected dimer 2, and the replaced *Ngo*M I/*Eco*R I internal fragment of the *SDH1* gene. This construct was used for all *frd* promoter yeast SDH operon expression studies.

II-9. Sequencing of the Synthetic Yeast SDH Operon (Plasmid pSdhOpF)

DNA sequence analysis, based on dideoxy chain termination method (124), was performed by the Department of Biochemistry DNA Core Laboratory (University of Alberta) using double stranded template DNA. The nucleotide sequence was determined for the entire open reading frame in one direction from either strand of DNA. The 1.5-kb *Ngo*M I/*Eco*R I region of the *SDH1* gene was not sequenced.

II-10. Construction of Plasmid pSdhOpT7: Placing the Synthetic Yeast SDH Operon Under the Control of the T7 Promoter

For use in expression studies using the T7 RNA polymerase/promoter expression system the synthetic yeast SDH operon was modified to place it under the control of the T7 bacteriophage promoter. A schematic diagram outlining the construction of the SDH operon under the bacteriophage T7 promoter is shown is Fig 2-4. The majority of fragment I was removed from the operon while maintaining the 13-bp directly upstream of the GTG start codon of the *SDH1* gene which contains the *E. coli* ribosome binding site. The T7 promoter was provided by the vector pBC KS$^-$.

Plasmid pPCRT7N Due to the absence of any convenient restriction endonuclease sites, construction of pPCRT7N, carrying an N-terminal fragment of *SDH1* (775-1216 of the SDH operon) under T7 control, was constructed as follows. A polymerase chain reaction was used to amplify a 460-bp region of *SDH1* from the operon in plasmid pSdhOpF. The primers used for PCR are listed in Table II-4. The upstream primer, SDH1D, was designed to add a *Sac* I restriction site 5′ to the ribosome binding site upstream of the GTG start codon of *SDH1*(ie. 5′ to nucleotide 775 of the operon). The downstream primer, SDH1C, was designed with a *Spe* I restriction site added 3′ to nucleotide 1216 of the operon. This primer was designed to be both far enough downstream of the *SDH1* GTG start codon as to produce a fragment that was an easy size to clone, and to produce a region of *SDH1* that included a restriction site unique to the operon (*Sac* II, nt 828-833) to facilitate cloning the remainder of the operon behind the new *SDH1*-T7 gene in one step. The new *Spe* I site that was created was not designed to be part of the new final operon under T7 control. PCR was performed using the Expand™ Long Template PCR System. The DNA was denatured for 30 sec at 94 °C, annealed for 30 sec at 65 °C, and extended for 1 min at 68 °C. The cycle was repeated 25 times.

The 460-bp PCR-generated fragment was cloned as a *Sac* I/*Spe* I fragment into the likewise digested vector pBC KS.

Plasmid pSdhOpT7 The remainder of the operon was cloned in behind the partial *SDH1* gene under T7 control, as a *Sac* II/*Bam*H I fragment from plasmid pSdhOpF into the *Sac* II/*Bam*H I sites of pPCRT7N. A clone demonstrating the correct restriction fragment pattern was further confirmed by sequencing from the *Sac* I site through to the *Sac* II site.

II-11. Expression Studies

II-11.a) Anaerobic Expression of the Synthetic Yeast SDH Operon From the Frd Promoter

The expression of the synthetic yeast SDH operon was carried out based on the method developed for overexpressing *E. coli* FRD with the following modifications (87). *E. coli* HB101 was transformed with the appropriate plasmids (pSdhOpF or pUC19e). Transformed cells were grown aerobically overnight with shaking at 37 °C in LB containing antibiotics. For induction of each time point, 2.5 mL of overnight aerobic preculture was used to innoculate 297.5 mL GF medium containing antibiotics and sealed. The 1% innuculum cultures were allowed to grow unopened at 37 °C with stirring until time of harvest. Samples were collected after the indicated number of hours of anaerobic growth. The optical density at 590 nm was measured and membrane and soluble fractions were prepared for SDS-PAGE gel analysis as described below.

II-11.b) Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter:

II-11.b) i. Selective Radiolabeling of Plasmid-Encoded Proteins

The selective labeling of plasmid-encoded proteins using the T7 promoter/T7 RNA polymerase system was carried out following the method described by Tabor and Richardson (137). *E. coli* K38/pGP1-2 was transformed with pSdhOpT7, pBC KS$^-$ (negative control), or neither and grown aerobically overnight with shaking at 30 °C in 5 mL LB + kan + cam. The overnight preculture was diluted 1:40 into 5 mL of fresh LB + kan + cam medium and grown aerobically at 30 °C to an optical density of approximately 0.4 at 590 nm. For labeling, 1.5 mL samples were harvested by centrifugation and washed twice with M9 medium. The washed cells were resuspended in 1 mL M9 supplemented with 1% each of Difco Methionine Assay Medium, thiamine, and glucose, and incubated for 90 min at 30 °C with shaking. Expression of the T7 RNA polymerase was induced by incubation at 42 °C for 20 min. Freshly prepared rifampicin was added to the cells (200 µg/mL) to inhibit host RNA polymerase and the cells were left at 42 °C for an additional 10 min. The cell suspensions were then shifted back to 30 °C for 30 min prior to labeling. To label newly synthesized proteins, 0.5 mL of cell suspension was removed to a scintillation vial and an aliquot of Trans^{35}S-Label (15 µCi) was added. In one set of controls, the radioactivity was omitted. After incubation for 8 min at 30 °C, the labeling reaction was stopped by the addition of 50 µl 100% TCA (10% final), mixing by inversion, and incubation on ice for 20 min. The precipitated proteins were harvested by centrifugation for 20 min (14,000 *g*) in a microcentrifuge at 4 °C. The pellet was washed twice with 600 µl acetone and resuspended in 40 µl 1x Tricine sample loading buffer for analysis by SDS-PAGE and audioradiography. 15 µl of the resuspended pellet ,which correspondes to 200 µl of original culture, was run per lane.

II-11.b) ii. Expression of Non-Radiolabeled, Plasmid-Encoded Proteins

Cells were grown as described above except precultures were diluted 1:40 into 100 mL fresh LB + kan + cam. The rapid temperature shift to 42 °C for induction of expression of the T7 RNA Polymerase was achieved by adding an equal volume (100 mL) of 55 °C medium. The culture was then shifted back to 30 °C after 30 min at 42 °C, and allowed to recover for 2 hrs with shaking before harvesting cells. Prior to harvest, a 1 mL aliquot of culture was collected and treated with 100 µl 100% TCA to obtain a whole cell sample comparable to the radiolabeled samples obtained from the T7 expression studies. The remaining 200 mL were harvested by centrifugation and used to prepare membrane and supernatant fractions for Western blot analysis.

II-12. Preparation of Membrane and Soluble Cellular Fractions

Cells were harvested by centrifugation at 10 000 *g* for 10 min and washed once in Buffer I (50 mM Tris-HCL, pH 7.5, 50 mM NaCl, 50 µg/mL PMSF, 1 mM EDTA, 1 mM benzamidine, 1 µg/mL leupeptin, 1 µg/mL pepstatin A), and resuspended in 3 mL Buffer I. Samples were kept on ice throughout the procedure. The cells were then lysed at 16, 000 psi by two passages through a French Pressure Cell. The cell lysates were centrifuged at a low speed spin (8,000 *g*) for 10 min in a Sorvall SS-34 rotor to remove cellular debris. The pellet containing cellular debris was collected and resuspended in 800 µl 50 mM Tris-HCl, pH 8.0. The supernatant was centrifuged at 95 000 *g* in a 100.3 rotor for 1 hr at 4 °C. The pellets from the high speed spin, containing the crude membrane fraction, were resuspended in 800 µl Buffer I. The supernatant of the high speed spin contains soluble proteins. All three fractions were frozen in aliquots in liquid nitrogen and stored at -70 °C until analysed by gel electrophoresis.

II-13. Determination of Protein Concentration

Protein concentrations were determined by a modified method of Bradford using the Bio-Rad Protein Assay kit following manufacturer's instructions (Bio-Rad Laboratories). BSA was used to create a standard curve.

II-14. Isolation of Mitochondria from Yeast

Mitochondria were isolated from *S. cerevisae* strain, D273-10B, Sdh1Ad1, or Sdh3W3 (see Table II-1 for genotype) as described by Gasser *et al.* for use as controls in immunoblots (37).

II-15. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

II-15.a) Laemmli SDS-PAGE

The Laemmli SDS-PAGE system was used to separate proteins by electrophoresis in 10 cm SDS mini-gels of 8-12% (w/v) acrylamide/bis-acrylamide (29:1), with a stacking gel of 4% acrylamide/bis-acrylamide using the BioRad Mini-Gel system apparatus (80). Samples were solubilized in Laemmli loading buffer, heated to 90 °C for 5 min, and then loaded on the gel. Gels were run at 80 V for 10 min, and for the remainder of the run at 120 V.

II-15.b) Tricine SDS-PAGE

The Tricine SDS-PAGE system was used for the separation of small proteins (i.e. <20 kDa) (131). Proteins were separated on a 10% (w/v) acrylamide/bis-acrylamide (29:1) gel with a 4% stacking gel. Samples were solubilized in Tricine loading buffer by heating to 40 °C for 30 min (unless otherwise stated) and loaded on the gel. Gels were run for 3 hr at 60 V.

II-16. Western Blot Anaylsis

For Sdh1p and Sdh2p detection, the Laemmli SDS-PAGE electrophoretic-transfer system was used, whereas for Sdh3p, the Tricine SDS-PAGE system was employed. Proteins separated on Laemmli gels were transferred to nitrocellulose membrane (Bio-Rad), while proteins separated on Tricine gels were transferred to PVDF membrane (Immobilon-P Transfer filters by Millipore). Transfers were performed using a semi-dry electrophoretic transfer process with a Tyler Research Transfer Cell. Nitrocellulose membrane-transfers were carried out at 200 mA for 30 min in transfer buffer (25 mM glycine, pH 8.3, 20% (v/v) methanol, 0.02% SDS). PVDF membrane-transfers were carried out at 60 mA for 3 hr using cathode buffer (300 mM 6-aminocaproic acid, 30 mM Tris, pH 8.6-8.7), and anode buffer (100 mM Tricine, 300 mM Tris, pH 8.7-8.8) (126). The membrane was then blocked overnight at 4 °C in Tris-buffered saline (TBS; 5 mM Tris base, 20 mM NaCl, pH 7.5) containing 10% powdered skim milk (w/v). After blocking, the membrane was washed 4 times with 15 mL TBS: 2x fast, 1x5 min, 1x10 min. The membrane was then incubated overnight with primary antibody diluted in TBS with 1% powdered skim milk at 4 °C. The primary antibody was saved for later reuse, and the membrane was washed as above. The secondary antibody, goat anti-rabbit antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.), was diluted 1:20,000 in TBS with 1% powdered skim milk and incubated with the membrane at room temperature for 1.5 hr. Detection was with the ECL system (Amersham).

II-17. Primary Antibodies

Sdh1p was probed with a 1:5,000 dilution of a rabbit polyclonal antiserum raised against a trpE-Sdh1p fusion protein consisting of 207 amino acids (Val-92 to Gln-298) of *Saccharomyces cerevisiae* Sdh1p, raised by Karen M. Robinson (109). The antiserum was affinity purified.

Sdh2p was detected using a rabbit polyclonal antiserum raised against a trpE-Sdh2p fusion prepared by Bernard Lemire. To purify the antiserum for use against *E. coli* hosts, the serum was reverse purified as follows. A 1:5,000 dilution aliquot of Sdh2p antiserum was repeatedly reacted to nitrocellulose bound to cell lysates of *E. coli*, strain HB101, harbouring the empty expression vector pUC19 until the background was satisfactorily reduced. This preparation was used for all subsequent probes and is referred to as Sdh2p antiserum.

Sdh3p was detected using a 1:1,000 dilution of a rabbit polyclonal antiserum raised against a peptide consisting of the amino 12 residues of the mature *Saccharomyces cerevisiae* Sdh3p prepared by S. Oyedotun (105).

II-18. Audoradiography Analysis

Gels containing ^{35}S-labelled proteins were fixed, treated with a flourography agent (Enhance^{TM} by Dupont), dried, and exposed to Kodak X-Omat film at -70 °C.

TABLE II-1: Strains Used In This Study

Strain	Relevant Genotype (Description)	Source or Reference
E. coli Strains		
DH5α	F′, ϕ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *rec*A1, *end*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1	(47)
GM48	*thr*, *leu*, *lac*Y, *gal*K, *gal*T, *ara*, *ton*A, *tsx*, *dam*, *dcm*, *sup*E44	(156)
GVK124	Δ(*lac-pro*AB), *hsd*5(r^-, m^-), *sup*E, *thi*F′, *lac*ZΔM15, *lac*1q, sdhC::kan, *rec*A;;Tn10	R. Gennis, Urbana Illinois
HB101	F', *hsdR*, *hsdM*, *pro*, *leu*, *gal*, *lac*, *thi*, *recA*, *rspL*	(123)
INVαF′	F′, *end*A1, *rec*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, ϕ80*lac*ZΔM15(*lac*ZYA*arg*F)U169λ-	Invitrogen Corporation
K38	HfrC, *phoA4*, *pit-10*, *tonA22*, *omp*F627, *rel*A1	(119)
S. cerevisiae Strains		
D273-10B	(MATα;ATCC 25657)	G. Schatz
Sdh1Ad1	MATα, *ura*3-del, *lys*2, rho+, *sdh1*::ADE2	(111)
Sdh3W3	MATα, *leu*2-3, *ura*3-52, *his*4, *his3*, *rme*, *sdh3*::TRP1	(105)

TABLE II-2: Plasmids and Phage Used in This Study

Plasmid or Phagemid	Relevant Characteristc	Source or Reference
p123R	Fragment I-VI subcloned from pBS123 into pUC19e with NgoM I/EcoR I::kan fragment replaced, amp^R	this study
pBCIVm	pBC KS, Fragment IV, cam^R	this study
pBC KS-	phagemid derived from pUC19, T7 promoter, cam^R	Stratagene
pBluescriptII SK+	phagemid derived from pUC19, amp^R	Stratagene
pBSd1	pBluescriptII, dimer 1, amp^R	this study
pBSd123	pBluescriptII, Fragment I-VI, amp^R	this study
pBSd2	pBluescriptII, dimer 2, amp^R	this study
pBSd3	pBluescriptII, dimer 3, amp^R	this study
pBSII-SDHB	pBluescriptIIKS-, *S. cerevisae SDH2*, amp^R	Lemire
pCR2.1	TA cloning vector, kan^R, amp^R	Invitrogen
pfrd79	pBR322, *Hin*d III fragment carrying the *E. coli frd* operon, amp^R	(26)
pGM205	pUC18, *S. cerevisae SDH3*, amp^R	(28)
pGP1-2	heat inducible T7 RNA polymerase gene under λp_L, c1857 under p_{lac} promoter, kan^R	(137)
pKIXX	pUC4-, kanamycin^{R}cassette, amp^R	Pharmacia Biotech
pPCRT7N	pBC KS-, N-terminus (460bp) of Fragment II under T7 promoter, cam^R	this study
pRA	pRS416, *S. cerevisae SDH1* gene, amp^R	(113)
pSDH4-17	pRS416, *S. cerevisae SDH4* gene, amp^R	(18)
pSdhOpF*	Correct synthetic yeast SDH operon (Fragment I-VI) cloned into pUC19e, amp^R	this study
pSdhOpT7*	pBC KS-, Fragments II-VI under the control of the T7 promoter, cam^R	this study
pTAIIIm	pCR2.1, Fragment III, kanR, amp^R	this study
pUC19	high copy number *E. coli* plasmid, amp^R	(156)
pUC19e	pUC19 with *Eco*R I site removed	this study
pUC19e123	Fragment I-VI subcloned into pUC19e, amp^R	this study
pUC19e123::kan	pUC19e123 with kan^R cassette disruption of Fragment II, amp^R	this study

* Plasmids used in expression studies

TABLE II-3: PCR Amplification of the SDH Operon Fragments

			PCR Cycle Parameters		
Fragment Generated	**Primer Pair**	**Plasmid Used As Template**	**Denature °C (sec)**	**Anneal °C (sec)**	**Extend °C (sec)**
I	SDH01 SDH02	pfrd79	94 (30)	48 (30)	68 (90)
II	SDH03 SDH04	pRA (linearized)	94 (30)	55 (30)	68 (135)
III	SDH05 SDH06	pBSII-SDHB	94 (30)	45 (30)	68 (150)
IV	SDH07 SDH08	pGM205	94 (30)	48 (30)	68 (90)
V	SDH09 SDH10	pSDH4-17	94 (30)	45 (30)	68 (60)
VI	SDH11 SDH12	pfrd79	94 (30)	45 (30)	68 (60)

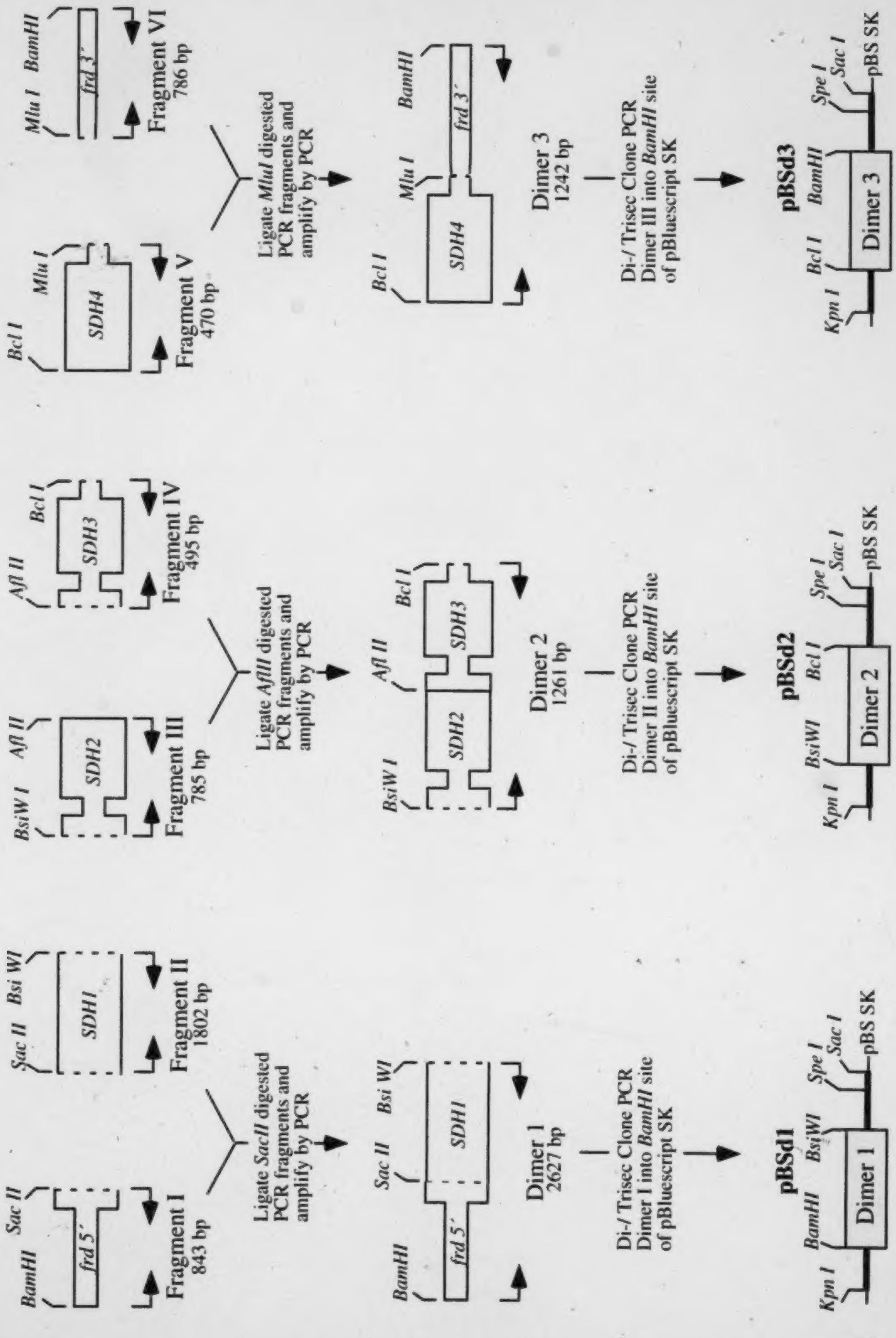
TABLE II-4: Sequence of Primers

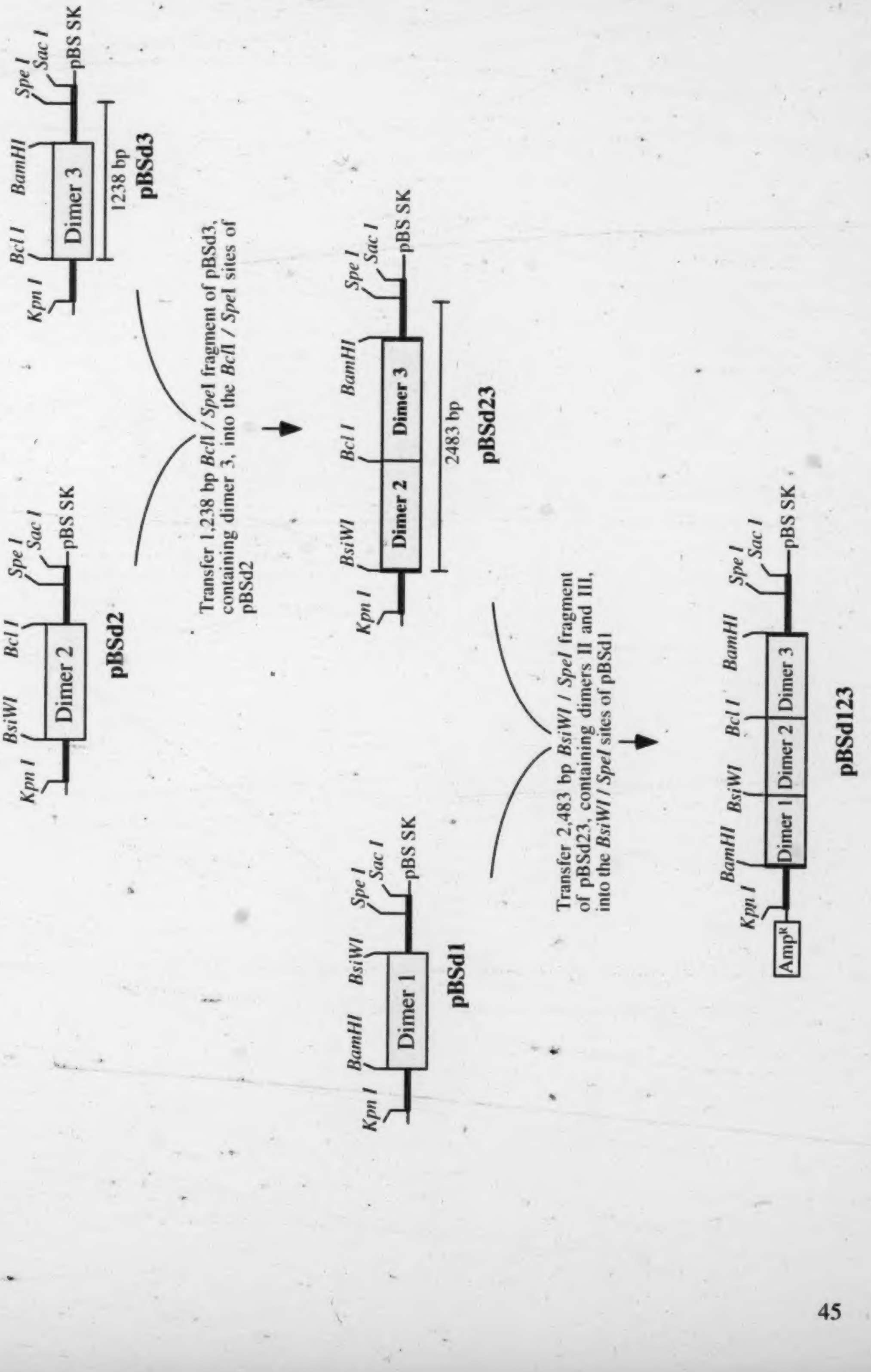
Primer Name	Sequence (5′ - 3′)	Characteristic Feature
Construction of the Synthetic Yeast Operon		
SDH01	atcgGGATCCAAGCTTGTTGATAAGAAAGG	*Bam*H I site
SDH02	atcgTTCCGCGGATCTGGACGCGGAACCGTTTACAGAGCCCTGGGTCTGCACGACATTCCTCCAG	*Sac* II site
SDH03	atcgTTCCGCGGACGGGAAGTACCACATAATAG	*Sac* II site
SDH04	atcgCGTACGGTTGGAGGTACGGAAGGAC	*Bsi*W I site
SDH05	atcgCGTACGAGCCTACTAAGGAGAAGGCGAATGGCTACTGCTACCACCGCTGCACGTACGCATAC	*Bsi*W I site
SDH06	atcgCCCTTAAGCAAATGCCAAAGATTTC	*Afl* II site
SDH07	atcgTTCTTAAGGAGTGCAACATGAACGTTGCTAGCGAAATGAACACCAAAGCG	*Afl* II site
SDH08	atcgCCTGATCATCTCAGGCTCCTTATAAAGTTAATAAATAAGTACC	*Bcl* I site
SDH09	atcgTTTGATCACTATCCCGTTCCTGCCGGTACTGCCGCAGAAACCAGGTGGTG	*Bcl* I site
SDH10	atcgACGCGTTACTTCTTGGCTTCAATC	*Mlu* I site
SDH11	atcgACGCGTCGCCAATGTAAATCCG	*Mlu* I site
SDH12	atcgGGATCCAAGCTTCAGCATCTAACG	*Bam*H I site
Construction of pPCRT7N		
SDH1D	ACTGAGCTCTGGAGGAATGTCGTGC	*Sac* I site
SDH1C	CGAACTAGTCTCCTTGGTCTGACC	*Spe* I site

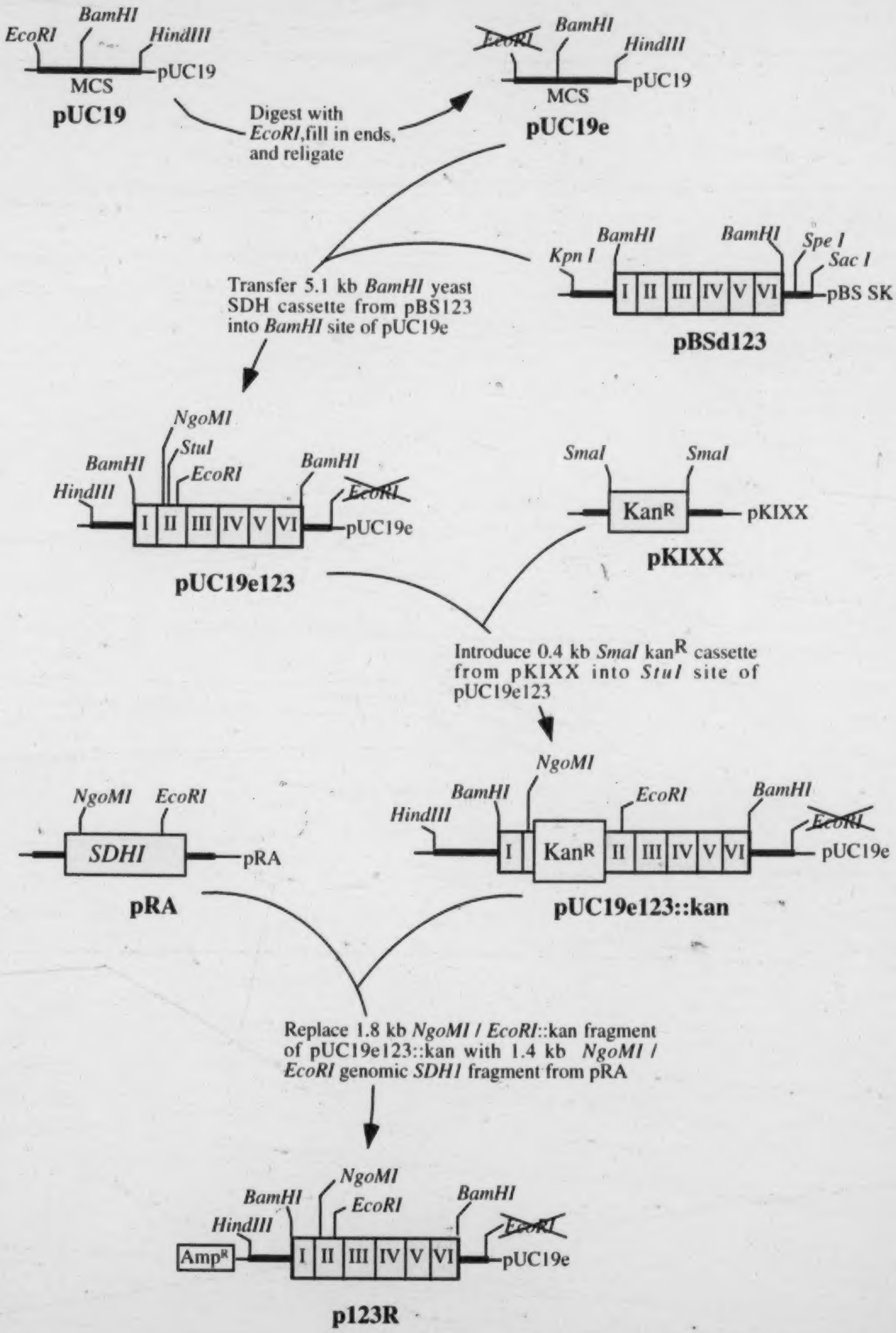
The underlined nucleotides mark the locations of the specified engineered restriction sites. The nucleotides represented in lower case letters were designed to facilitate Di/Trisec cloning.

TABLE II-5: PCR Amplification of Dimers

		PCR Cycle Parameters		
Fragment Generated	**Primer Pair**	**Denature °C (sec)**	**Anneal °C (sec)**	**Extend °C (sec)**
Dimer #1	SDH01 SDH04	94 (30)	55 (30)	68 (120)10x 68 (150) 5x 68 (180) 5x 68 (210) 5x
Dimer #2	SDH05 SDH08	94 (30)	60 (30)	68 (120)
Dimer #3	SDH09 SDH12	94 (30)	56 (30)	68 (120)


BamHI
Sac II
frd 5´
Fragment I
843 bp
Sac II
Bsi WI
SDH1
Fragment II
1802 bp
Ligate SacII digested PCR fragments and amplify by PCR
BamHI
Sac II
Bsi WI
frd 5´
SDH1
Dimer 1
2627 bp
Di-/ Trisec Clone PCR Dimer I into BamHI site of pBluescript SK
pBSd1
Kpn I
BamHI
BsiWI
Spe I
Sac I
Dimer 1
pBS SK
BsiW I
Afl II
SDH2
Fragment III
785 bp
Afl II
Bcl I
SDH3
Fragment IV
495 bp
Ligate AflII digested PCR fragments and amplify by PCR
BsiW I
Afl II
Bcl I
SDH2
SDH3
Dimer 2
1261 bp
Di-/ Trisec Clone PCR Dimer II into BamHI site of pBluescript SK
pBSd2
Kpn I
BsiWI
Bcl I
Spe I
Sac I
Dimer 2
pBS SK
Bcl I
Mlu I
SDH4
Fragment V
470 bp
Mlu I
BamHI
frd 3´
Fragment VI
786 bp
Ligate MluI digested PCR fragments and amplify by PCR
Bcl I
Mlu I
BamHI
SDH4
frd 3´
Dimer 3
1242 bp
Di-/ Trisec Clone PCR Dimer III into BamHI site of pBluescript SK
pBSd3
Kpn I
Bcl I
BamHI
Spe I
Sac I
Dimer 3
pBS SK


Kpn I
BsiWI
Dimer 2
Bcl I
Spe I
Sac I
pBS SK
pBSd2
Kpn I
Bcl I
Dimer 3
BamHI
Spe I
Sac I
pBS SK
1238 bp
pBSd3
Transfer 1,238 bp BclI / SpeI fragment of pBSd3, containing dimer 3, into the BclI / SpeI sites of pBSd2
Kpn I
BsiWI
Dimer 2
Bcl I
Dimer 3
BamHI
Spe I
Sac I
pBS SK
2483 bp
pBSd23
Kpn I
BamHI
Dimer 1
BsiWI
Spe I
Sac I
pBS SK
pBSd1
Transfer 2,483 bp BsiWI / SpeI fragment of pBSd23, containing dimers II and III, into the BsiWI / SpeI sites of pBSd1
AmpR
Kpn I
BamHI
Dimer 1
BsiWI
Dimer 2
Bcl I
Dimer 3
BamHI
Spe I
Sac I
pBS SK
pBSd123

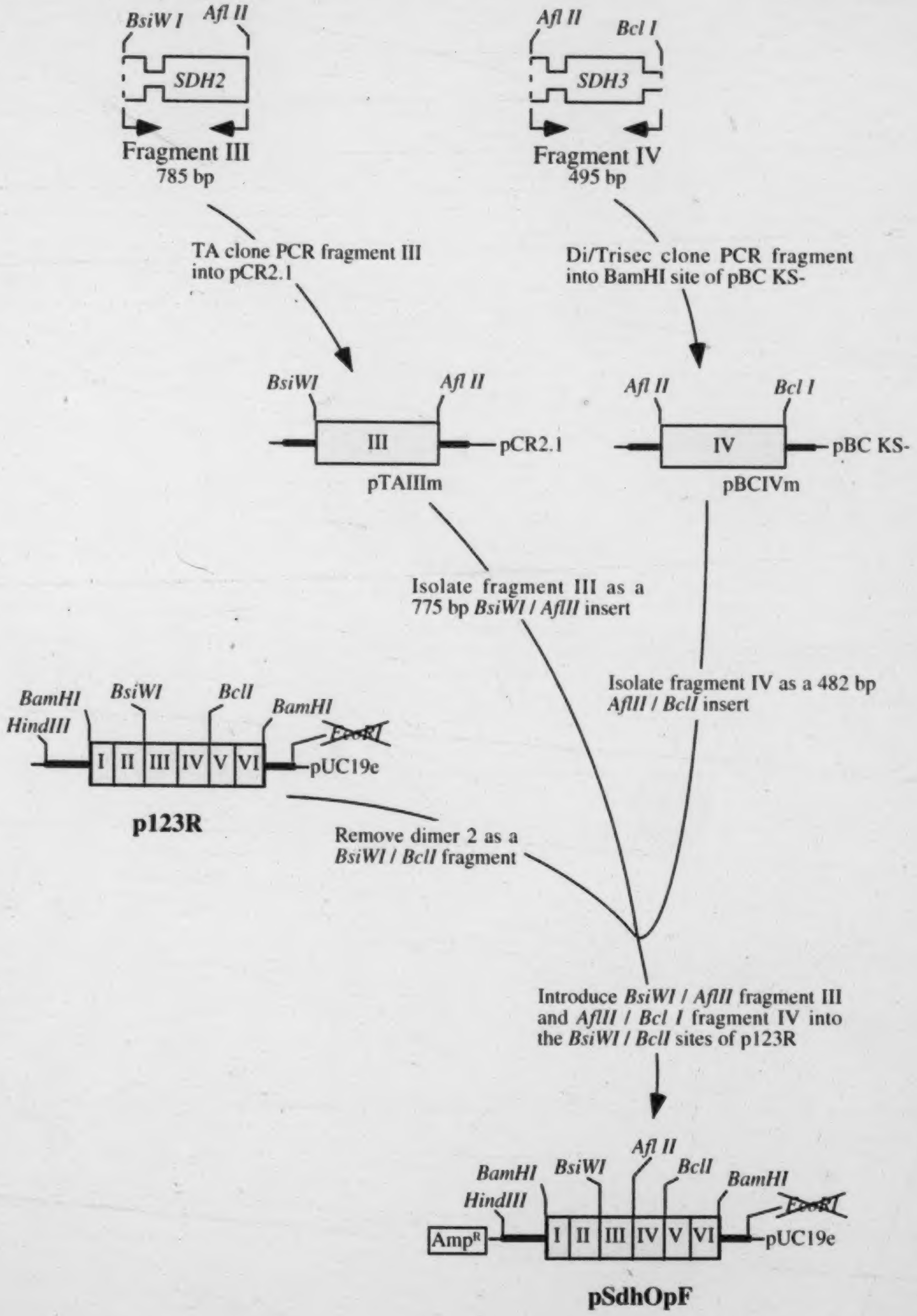


Digest with *EcoRI*, fill in ends, and religate

Transfer 5.1 kb *BamHI* yeast SDH cassette from pBS123 into *BamHI* site of pUC19e

Introduce 0.4 kb *SmaI* kan^R cassette from pKIXX into *StuI* site of pUC19e123

Replace 1.8 kb *NgoMI* / *EcoRI*::kan fragment of pUC19e123::kan with 1.4 kb *NgoMI* / *EcoRI* genomic *SDH1* fragment from pRA



TA clone PCR fragment III into pCR2.1

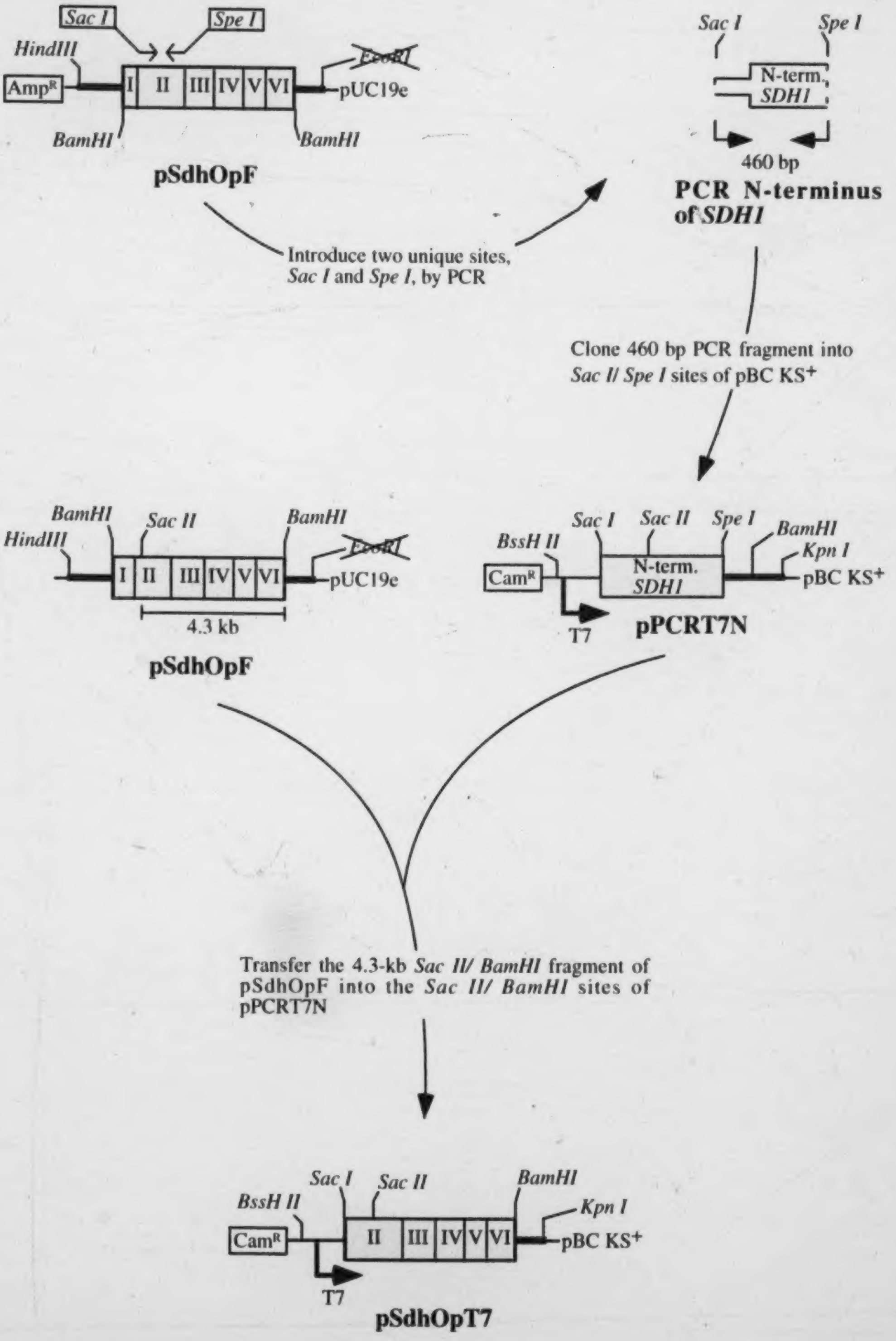
Di/Trisec clone PCR fragment into BamHI site of pBC KS-

Isolate fragment III as a 775 bp *BsiWI* / *AflII* insert

Isolate fragment IV as a 482 bp *AflII* / *BclI* insert

Remove dimer 2 as a *BsiWI* / *BclI* fragment

Introduce *BsiWI* / *AflII* fragment III and *AflII* / *Bcl I* fragment IV into the *BsiWI* / *BclI* sites of p123R



Introduce two unique sites, *Sac I* and *Spe I*, by PCR

Clone 460 bp PCR fragment into *Sac I/ Spe I* sites of pBC KS$^+$

Transfer the 4.3-kb *Sac II/ BamHI* fragment of pSdhOpF into the *Sac II/ BamHI* sites of pPCRT7N

Chapter III: Expression of Yeast SDH in *E. coli*; Rationale and Design

III-1. Introduction

The major goal of my research was to develop an *E. coli* expression system for the *S. cerevisiae* SDH complex in order to produce enough functional protein for biophysical and mutational studies. Our knowledge of the structural and functional characteristics of this cofactor-containing complex would benefit greatly from such studies. Attempts to overexpress the complex in yeast have not been successful. The approach I adopted to obtain high levels of functional yeast SDH was to design and construct a synthetic yeast SDH operon for bacterial expression. The design and rationale for this operon are based on the highly efficient overexpression system for the closely related *E. coli* enzyme, FRD.

E. coli FRD and yeast SDH are very similar in composition and structure even though they catalyse the opposite enzymatic reactions *in vivo*. They are both composed of a membrane-peripheral catalytic domain containing a flavoprotein subunit (FrdA or Sdh1p; 70 kDa) with one covalently bound FAD, and an iron-sulfur protein subunit (FrdB or Sdh2p; 27 kDa) containing three iron-sulfur clusters, and a membrane-integral domain consisting of two anchor subunits (FrdC or Sdh3p; 17 kDa, and FrdD or Sdh4p; 13 kDa). Thus, they both have four subunits that require the insertion of similar cofactors, and likely have similar assembly requirements. *E. coli* FRD can be functionally overproduced 30-40 fold in *E. coli*. This demonstrates that *E. coli* is able to handle the cofactor insertion and membrane assembly of excessive amounts of FRD. Since SDH is so similar, *E. coli* may be able to likewise overproduce the yeast SDH.

FRD is encoded in the *E. coli* genome as an operon. Overexpression of the *E. coli* FRD complex was achieved by cloning a 4.9-kb *Hind* III DNA fragment containing the *frd* operon into the multicopy plasmid, pBR322, producing the plasmid pfrd79. Induction of the plasmid-encoded operon under anaerobic conditions leads to overproduction (86). Therefore, we decided to express the yeast SDH in *E. coli* by creating a synthetic yeast SDH operon based on the architecture of the *E. coli frd* operon (Figure III-1.).

The *E. coli frd* operon is arranged as *frdABCD* and is under the control of a single promoter. The promoter is induced under anaerobic conditions on a non-fermentable carbon source such as glycerol or lactate, with fumarate as the ultimate electron acceptor. The *frd* operon displays several unusual genetic features (26). Firstly, the *frdA* gene employs the less commonly used GTG initiation codon. Secondly, the operon displays extreme sequence economy. The intergenic regions are atypically short with the translation stop codons being incorporated into the ribosome binding sites of the following gene, and there is an 8 bp overlap between the *frdA* and *frdB* genes. All of these features have been highly conserved in *Enterobacteriaceae frd* operons as shown by DNA sequence analysis (26).

As it is not understood which elements of the *frd* operon are specifically responsible for enabling massive overexpression, the molecular organization of the synthetic yeast SDH operon was designed to mimic that of the *E. coli frd* operon as closely as possible. Essentially the open reading frames encoding the Frd subunits were replaced with the open reading frames encoding mature yeast SDH subunits (Figure III-1).

In general, the synthetic yeast SDH operon consists of the 5′ *frd* operon control elements, followed by the open reading frames encoding the mature yeast SDH subunits in the same order as they appear in the *frd* operon (i.e. *SDH1*, *2*, *3*, and then *4*), and terminates with the *frd* operon 3' sequence. The short intergenic regions of the *frd* operon, containing the *E. coli* ribosome binding sites, were inserted between the SDH genes. The DNA sequences upstream and downstream of the SDH operon are the same as in the plasmid pfrd79. The roles of the upstream and downstream sequences in FRD overexpression are not fully defined. To avoid the loss of an important regulatory element, we decided to retain these sequences as found in plasmid pfrd79.

In yeast, each of the four SDH subunits is synthesized with a targeting signal that directs the subunit into the mitochondria for assembly. These targeting signals are proteolytically removed prior to enzyme assembly. For expression in *E. coli*, which does not contain intracellular compartments, the targeting signals are unnecessary and may even impair assembly if included. For the construction of the SDH operon, the targeting signals were replaced by initiator methionine codons.

Translation of a foreign message can be limited in *E. coli* because of differences in codon usage between prokaryotes and eukaryotes (44, 74). It has been shown that the base composition of the first ten codons can be important for efficient expression, while the codon usage for the rest of the gene is less important (129). Therefore, any low-usage *E. coli* codons within the first ten codons of each gene of the SDH operon were altered to more preferred codons without modifying the encoded protein product.

Finally, unique restriction sites were designed between the SDH genes of the operon (Figure III-1) to both facilitate the construction of the operon, and to enable "cassette insertion" of mutant versions of any one of the individual SDH genes. The ability to easily replace wildtype genes with mutant genes and compare functional phenotypes will increase the value of this system as a tool.

Following, is a detailed description of the synthetic yeast SDH operon.

III-2. Detailed Description of the Synthetic Yeast SDH Operon

The synthetic yeast SDH operon is a 5.1-kb fragment of DNA that places all four SDH genes under the control of the *E. coli frd* promoter. The design of the operon is based on the molecular organization of the *E. coli frd* operon (Figure III-1). Following is a description of nucleotides 1-5090 of the synthetic yeast SDH operon. The nucleotide sequences at the junctions between Fragments I-VI are shown in Figures III-2 to Figure III-8, respectively.

Nucleotides 1-6 and 5085-5090 encode two flanking *Bam*H I restriction sites that were designed to allow for the removal of the entire SDH operon as a 5.1-kb *Bam*H I DNA fragment (Figures III-2 and III-8).

Nucleotides 7-787 consist of the DNA sequence stretching from -781 to -1 of the *frd* operon where +1 is the first nucleotide of the FrdA open reading frame (Figures III-2 and III-3). This sequence places the synthetic yeast SDH operon under the control of the *frd* promoter. The *frd* promoter is located some 93 nucleotides upstream of the *frdA* coding sequence as judged by S1 nuclease mapping and primer extension analysis of mRNA (26). The additional sequence to the upstream *Hind* III site (-93 to -781) was included to parallel the situation in plasmid pfrd79.

Nucleotides 788-2629 encode the *S. cerevisiae* Sdh1p subunit from Gln-29 to Tyr-640 with a GTG start codon added to the N-terminus immediately preceding Gln-29 of Sdh1p (Figures III-3 and III-4). In yeast, the Sdh1p precursor protein contains an N-terminal mitochondrial targeting sequence that is cleaved following import to form the mature protein. The Sdh1p precursor polypeptide contains the consensus sequence for cleavage to a mature species by two separate proteases: RX(F)XX(S) where R is arginine, X is any amino acid, (F) is phenylalanine or any hydrophobic residue, and (S) is serine, threonine, or glycine (58). The first protease is predicted to cleave between Thr-20 and Phe-21, and the second between Arg-28 and Gln-29 to form the mature species. The amino-terminal residue in mature Sdh1p was determined to be a glutamic acid instead of a glutamine (18). The significance of this finding is not clear. The less commonly used GTG initiation codon of *frdA* is one of several unusual genetic features that has been highly conserved during the evolution of the *frd* operon (26). Therefore, it was decided to employ it instead of the more common ATG initiation codon for *SDH1* in the yeast SDH operon. Changes to the yeast *SDH1* DNA sequence, not affecting the amino acid sequence include (Figures III-3 and III-4): i) two nucleotides within the first ten codons substituted to accommodate the codon preferences specific to *E. coli*, ii) two bases changed to create a unique *Sac* II restriction site and, iii) two nucleotides changed to create a unique *Bsi*W I restriction site.

Nucleotides 2630-2640 are the intergenic region between *SDH1* and *SDH2* (Figure III-4). These 11-bp are identical to the 11-bp that immediately preceed the ATG of *frdB* in the *frd* operon. In the *frd* operon there is no intergenic region between the *frdA* and *frdB* genes. Instead, there is an 8-bp overlap between the *frdA* and *frdB* coding region such that the ribosomal binding site for *frdB* is located within the open reading frame of *frdA*. This extreme sequence economy is another characteristic feature of the *frd* operon that is highly conserved. For the SDH operon it was not possible to duplicate the overlap without changing the amino acid sequences of either Sdh1p or Sdh2p. As this was considered undesirable, a synthetic intergenic region was created.

Nucleotides 2641-3387 encode Met-19 to Ala-266 of the *S. cerevisiae* Sdh2p (Figures III-4 and III-5). For expression in *E. coli*, the first eighteen amino acids, comprising the leader sequence of the precursor protein, are not included in the SDH operon sequence. The site of presequence cleavage for Sdh2p has not been experimentally verified by N-terminal protein sequencing. Three nucleotides of three codons within the first ten codons were substituted to accommodate *E. coli* codon usage preferences, and two nucleotides were altered to create an unique *Afl* II restriction site. Met-19 of the yeast Sdh2p sequence served as the initiation codon.

Nucleotides 3388-3397 are the intergenic region between *SDH2* and *SDH3* of the SDH operon (Figure III-5). These 10-bp are identical to the intergenic 10-bp between *frdB* and *frdC* of the *frd* operon.

Nucleotides 3398-3847 encode Asn-51 to Leu-198 of the *S. cerevisiae* Sdh3p (Figures III-5 and III-6). The N-terminal fifty amino acids comprising the mitochondrial targeting sequence (28) were replaced with an ATG start codon. In addition to this change to *SDH3*, four bases within four codons of the first 10 codons were altered to create preferred *E. coli* codons.

Nucleotides 3848-3857 are the intergenic region region between *SDH3* and *SDH4* in the SDH operon (Figure III-6). These 10-bp are identical to the intergenic region between *frdC* and *frdD* in the *frd* operon.

Nucleotides 3858-4313 encode Leu-32 to Lys-181 of yeast Sdh4p (Figures III-6 and III-7). In Sdh4p, the first thirty one amino acid residues comprise the mitochondrial targeting sequence (18) and were not included. For expression in *E. coli*, the start codon, ATG, was added upstream of Leu-32. Leu-32 was changed to Ile-32 by the substitution of one base in order to create a unique *Bcl* I restriction site. Other restriction sites were considered but no other convenient cloning restriction site could be designed in this region without altering the amino acid composition of the polypeptide. Changing Leu to Ile is considered a conservative change that should have minimal effects on the structure or function of the polypeptide. Seven bases within six codons of the first 10 codons were changed to accommodate the codon preferences of the expression host.

The remaining nucleotides 4314-5085 consist of the *frd* operon terminator sequence that follows immediately downstream of *frdD* to, and including, the *Hind* III site (Figure III-8). Of this sequence, one base (4317) was changed to create an *Mlu* I restriction site. A *Bam*H I site was added on to the 3′ end (5085-5090).

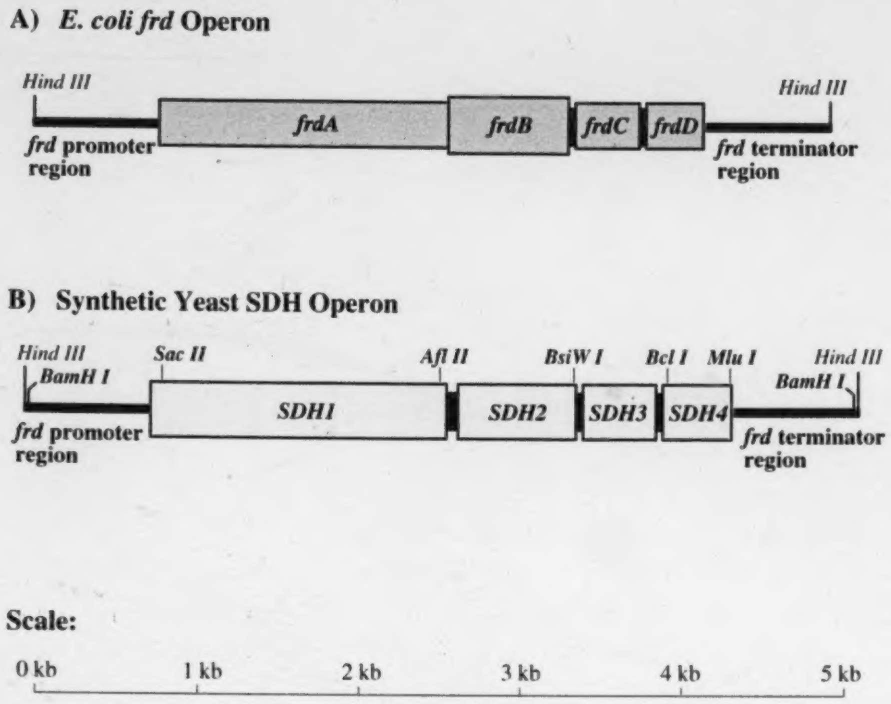


Figure III-1. Organization of the *E. coli frd* Operon and the Synthetic Yeast SDH Operon

A map of the *E. coli frd* operon (A), and the synthetic yeast SDH operon (B) is shown. The diagram is drawn to scale with the exception of the intergenic regions. The direction of transcription for each operon is from left to right as drawn. A) Structure of the *E. coli frd* operon. The 4.9 kb *Hind* III fragment containing the *frd* operon is shown. The four genes *frdA*, *frdB frdC*, and *frdD* are under the control of a single anaerobic-inducible promoter. The open reading frame of the *frdA* and *frdB* genes overlap. The intergenic regions between *frdB/frdC* and *frdC/frdD* are 10 and 11 bp in length, respectively.
B) Design of the Synthetic Yeast SDH Operon. The SDH operon mimics the *frd* operon in design as closely as possible. The genes for the FRD subunits have been replaced with those for the mature yeast SDH subunits. An overlap between the *SDH1* and *SDH2* genes was not reproduced. Six restriction sites (as shown in bold) were engineered to facilitate construction and enable "cassette insertion" of SDH subunit mutants.

Fragment I: 5′ End

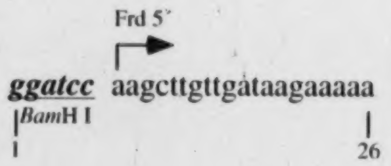


Figure III-2. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides1-26

This figure shows the sequence of the synthetic yeast SDH operon in area where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Fragment I - Fragment II: Junction

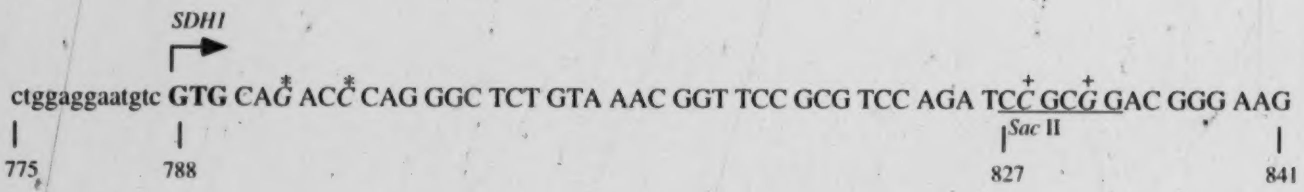

SDH1
ctggaggaatgtc GTG CAG ACC CAG GGC TCT GTA AAC GGT TCC GCG TCC AGA TCC GCG GAC GGG AAG
775
788
Sac II
827
841

Figure III-3. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 775-841

This figure shows the sequence of the synthetic yeast SDH operon in areas where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Fragment II - Fragment III: Junction

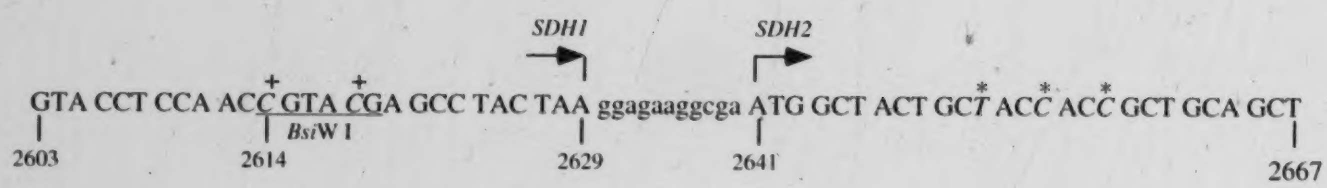

SDH1
SDH2
GTA CCT CCA ACC GTA CGA GCC TAC TAA ggagaaggcga ATG GCT ACT GCT ACC ACC GCT GCA GCT
+
+
*
*
*
BsiW I
2603
2614
2629
2641
2667

Figure III-4. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 2603-2667

This figure shows the sequence of the synthetic yeast SDH operon in areas where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Fragment III - Fragment IV: Junction

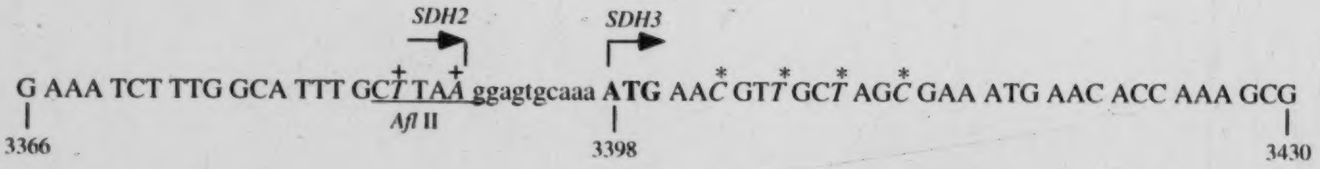

SDH2
SDH3
G AAA TCT TTG GCA TTT GCT TAA ggagtgcaaa ATG AAC GTT GCT AGC GAA ATG AAC ACC AAA GCG
+
+
*
*
*
*
Afl II
3366
3398
3430

Figure III-5. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 3366-3430

This figure shows the sequence of the synthetic yeast SDH operon in areas where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Fragment IV-Fragment V: Junction

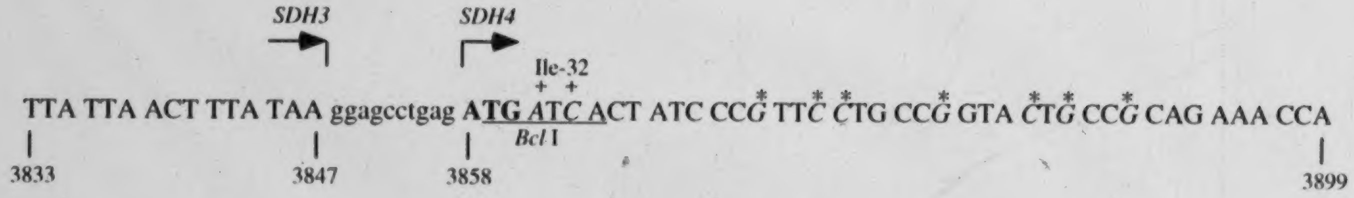

SDH3
SDH4
Ile-32
+ +
* * * * * * *
TTA TTA ACT TTA TAA ggagcctgag ATG ATC ACT ATC CCG TTC CTG CCG GTA CTG CCG CAG AAA CCA
BclI
3833
3847
3858
3899

Figure III-6. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 3833-3899

This figure shows the sequence of the synthetic yeast SDH operon in area swhere changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning". Ile-32 replaces Leu-32 in the native yeast Sdh4p sequence.

Fragment V - Fragment VI: Junction

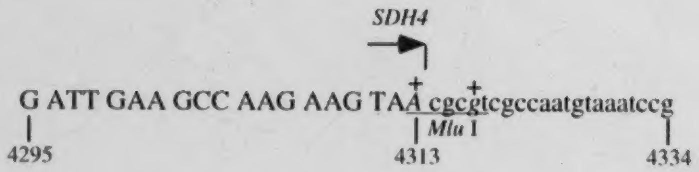


Figure III-7. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 4295-4334

This figure shows the sequence of the synthetic yeast SDH operon in areas where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Fragment VI: 3′ End

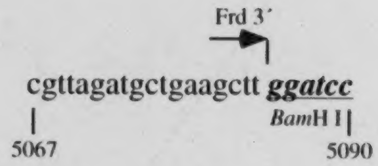


Figure III-8. Detailed nucleotide sequence of the Synthetic Yeast Operon at the junctions between the SDH and FRD-derived DNA: nucleotides 5067-5090

This figure shows the sequence of the synthetic yeast SDH operon in areas where changes were required. The numbers below the sequence refer to the nucleotide positions 1 - 5090 of the yeast SDH operon. Nucleotides in upper case letters are derived from *S. cerevisiae* SDH genes. Nucleotides represented as lower case letters are derived from the *E. coli frd* operon. Nucleotides in bold have been inserted into the operon. Nucleotides in italics have been altered from parental DNA. Italic nucleotides marked with a plus sign (+) have been changed to create a restriction site, whereas those with an asterisk (*) have been changed to accommodate *E. coli* codon preferences. Underlined nucleotides indicate the site where a unique endonuclease restriction recognition sequence was engineered to allow "cassette cloning".

Chapter IV: Results

IV-1. Introduction

We sought to develop an expression system to functionally overproduce the *S. cerevisiae* SDH in *E. coli.* We attempted to do so by designing and constructing an operon encoding all four mature yeast subunits arranged for expression in *E. coli.* The design and rationale for the yeast SDH operon were based on the *E. coli frd* operon. *E. coli* FRD and yeast SDH are both structurally and functionally very similar enzymes. They both consist of four nonidentical subunits that require the insertion of similar cofactors, and that must assemble and be targetted into a membrane. The *E. coli* FRD has been successfully overproduced thirty to forty fold by cloning the *frd* operon into a multicopy plasmid and inducing expression under anaerobic conditions (86). The strategy to express the yeast SDH in *E. coli,* consisted of creating a yeast SDH operon that mimicked the *frd* operon as closely as possible, and similarly inducing expression anaerobically.

In this chapter, I present the successful construction of the synthetic yeast SDH operon. Expression of the SDH operon was tested in the *E. coli* strain HB101 grown in glycerol-funarate (GF) medium under anaerobic growth conditions. Yeast polypeptides were identified using antisera raised against Sdh1p, Sdh2p, or Sdh3p. No antiserum against Sdh4p has been successfully produced. Expression of the SDH operon was additionally tested in the *E. coli* strain, K38/pGP1-2, where the operon was placed under the control of the T7 promoter provided by the vector, pBC KS-. ^{35}S-labeled amino acids were used to specifically label plasmid-encoded polypeptides.

IV-2. Construction of the Synthetic Yeast SDH Operon

The design of the synthetic yeast SDH operon was described in Chapter 3. Construction of the SDH operon was as described in Chapter 2: Materials and Methods. The sequence of the SDH operon, contained in the plasmid pSdhOpF, was confirmed by sequencing the entire open reading frame in one direction. Two PCR-generated mistakes were detected, corrected, and the new sequence confirmed. The 1.5-kb *NgoM I/EcoR* I region of the *SDH1* gene was not sequenced, as it is of genomic origin.

IV-3. Expression Studies

IV-3.a) Anaerobic Expression of the Synthetic Yeast SDH Operon From the frd Promoter

We attempted to express all four mature subunits of the yeast SDH in *E. coli* using the expression plasmid pSdhOpF containing the synthetic yeast SDH operon under the control of the *E. coli frd* promoter. This construct should direct expression of all four mature SDH polypeptides when induced by anaerobic conditons on a non-fermentable carbon source such as GF. We expect the heterologous host to be able to properly modify the yeast polypeptides by adding the required cofactors, to target the anchor subunits to the cytoplasmic (inner) membrane, and to assemble the subunits into a functional complex. The selection of the expression host strain, and the growth and induction conditions for expressing the synthetic yeast SDH operon, were based on the expression protocol as originally developed for the overexpression of the *E. coli* FRD (86). The original protocol was modified to include a time course of expression. A 1% innoculum of pSdhOpF-transformed HB101 cells was induced by anaerobic growth in glycerol fumarate medium. Cells were harvested, lysed, separated into membrane and soluble fractions, and run on an SDS-polyacrylamide gel. Yeast polypeptides produced in *E. coli* were visualized by Western blotting using antisera against Sdh1p, Sdh2p, and Sdh3p. As a negative control for the presence of non-specific immunoreactive proteins, HB101 transformed with the empty expression vector, pUC19e, was grown and harvested 48 hr after introduction into inducing GF medium. As a control for the yeast SDH antisera cross-reacting with the equivalent *E. coli* SDH subunit, an *E. coli* SDH knockout strain, GVK124, was also included. As antisera controls, mitochondria were isolated from wildtype or knockout strains of *S. cerevisiae* and run in adjacent lanes to show the expected migrations in SDS of the SDH subunits. Expression was studied during a broad time course spanning 3 days, and then in a subsequent, more focussed time course.

The long time course examined expression at 12 hr, 24 hr, 48 hr, 60 hr, and 72 hr after introduction into inducing GF medium. We did not determine when anaerobiosis was reached.

The Western blots probed with the anti-Sdh1p antiserum did not reveal any convincing immunoreactive bands of the expected size for any of the time points examined in either the membrane or soluble fractions (Figure IV-1). In Figure IV-1, panel A, the positive control lane containing wildtype mitochondria shows that the expected apparent size of mature Sdh1p is 67-kDa (lane 1). For the 24 and 48 hr time points of the membrane fraction, there are two faint immunoreactive bands migrating near 38-kDa that are not present in the negative HB101/pUC19e control which may represent degradation products (Figure IV-1, panel A, lanes 6, 7). For the gel of the soluble fractions, the positive control lane (Figure IV-1, panel B, lane 1), consisting of wildtype yeast mitochondria, contained BSA in the sample. The presence of BSA results in Sdh1p migrating faster than usual on this gel. There is a band at the expected apparent molecular mass in the 12 through 72 hr time points in the soluble fractions; however, it is also in HB101/pUC19e (Figure IV-1, panel B, lane 4). Thus, we considered it to be non-specific. It is not *E. coli* SdhA because the band is present in the *E. coli* SDH-knockout sample (Figure IV-1, panel B, lane 3).

Antiserum against Sdh2p identified a band of the expected size in the membrane fraction of the 12 hr time point (Figure IV-2, panel A, lane 5). As expected, this 28-kDa immunoreactive band is not present in the negative lane, HB101/pUC19e (lane 4). The positive control lane, containing wildtype mitochondria, shows that the expected apparent size of mature yeast Sdh2p is 28-kDa (Figure IV-2, lane 1). The band was not detectable in later time points or in the soluble fractions (Figure IV-2). Expression levels of Sdh2p at 12 hr were considered to be low.

In agreement with the Sdh2p results, antisera against Sdh3p detected a very strong immunoreactive species in the membrane fraction of the 12 hr time point (Figure IV-3, panel A, lanes 5, 9). The immunoreactive species was migrating with an apparent molecular mass of 37-kDa instead of the expected 14-kDa. In the Tricine SDS-PAGE gel system, the mature Sdh3p subunit (16, 675-Da) migrates at an apparent molecular weight of 14-kDa, while the mature Sdh4p subunit (16, 638-Da) migrates less rapidly with an apparent molecular weight of 17-kDa(17). We failed to detect Sdh3p in the positive control mitochondrial samples. As noted earlier in the lab (Oyedotun, unpublished results), the anti-Sdh3p serum fails to detect Sdh3p resolved on Laemmli gels. It was found that Sdh3p could be detected after separation on a Tricine-SDS polyacrylamide system, and transfer to a PVDF membrane (Oyedotun, unpublished results). This gel system was used for the samples of the short time course and will be presented below.

In summary, results from the 72 hr time course indicated low accumulations in the membrane fractions of expressed yeast Sdh2p and possibly Sdh3p at the 12 hr time point. A second time course was performed to test expression at 10 hr, 13 hr, and 18 hr following induction of the *frd* promoter. Samples from the cell debris fraction were also included on the gels.

Sdh1p, again, could not be detected (Figure IV-4). In the membrane and cell debris fractions there is background immunoreactivity in the area of interest that may interfere with the detection of Sdh1p. Weak expression of Sdh2p was detected equally in the membrane fractions of time points 10, 13, and 18 hr. (Figure IV-5, lanes 8, 9, 10).

Expression of Sdh3p during the short time course was tested using a Tricine-SDS PAGE gel system (Figure IV-6). In this system, Sdh3p is detected in the positive mitochondrial control at the expected size of 14-kDa (Figure IV-6, lane 2). In agreement with the Sdh3p results obtained from the Laemmli-SDS PAGE system, an immunoreactive band is detected in the membrane fraction, again migrating at an apparent molecular mass of 37-kDa. The band can be seen in the 10, 13, and 18 hr time points of the membrane fractions (Figure IV-6, lanes 8, 9, 10). No immunoreactive species was detected at the expected size of 14-kDa on the Tricine-SDS system. In addition, membrane samples were prepared for loading on the Tricine-SDS gel by alternatively heating at 40 °C for 30 min, 100 °C for 10 min, or 80 °C for 10 min to determine whether the method of sample preparation affected the apparent molecular weight on the gel. No differences in the migration were observed (data not shown).

These data indicate that anaerobic expression under the *frd* promoter is limited or that the subunits produced are unstable. Of the three available antisera, only the anti-sdh2 serum detected a product of the expected size. A strong Sdh3p immunoreactive species was detected, but was of an unexpected size. The reason for this is not known. Interestingly, both subunits were detected solely in the membrane fractions. Sdh2p is a soluble protein, whereas Sdh3p is an integral membrane protein. When assembled into an enzyme complex, the soluble catalytic dimer composed of Sdh1p and Sdh2p, becomes associated with the membrane through interaction with the anchor subunits, Sdh3p and Sdh4p. While it is encouraging to detect Sdh2p in the membrane fraction, it is not conclusive evidence of enzyme assembly. Thus, while consistent with assembly, better evidence of the expression of mature yeast Sdh3p and Sdh4p is needed. No accumulation of expressed yeast Sdh1p was detected. Failure to observe expression could not be ascribed to the plasmid construct, as it had been confirmed by sequencing. The possibility that the mRNA or the translated protein were toxic to the *E. coli* could also not be supported as the *E. coli* grew as well as control *E. coli*. Expression levels were too low to make studies of cofactor attachment or enzyme activity feasible.

IV-3.b) Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 RNA Polymerase Promoter

In light of the failure to identify the expression of mature *S. cerevisiae* Sdh1p using antibodies, the synthetic yeast SDH operon was placed under the control of the bacteriophage T7 promoter for study using the T7 RNA polymerase/promoter expression system as described by Tabor and Richardson (137). This system was considered advantageous for several reasons. The T7 RNA polymerase is a very active enzyme and synthesizes RNA at five times the rate of *E. coli* RNA polymerase. In addition, the T7 RNA polymerase is highly selective for initiation at its own promoter sequence and it does not initiate transcription from any sequence on the *E. coli* DNA (136). Finally, it is resistant to antibiotics such as rifampicin that inhibit the *E. coli* RNA polymerase, and consequently, the addition of rifampicin to cells that are producing T7 RNA polymerase results in the exclusive expression of genes under the control of a T7 promoter. These can then be exclusively labeled with ^{35}S-labeled amino acids. This is the most relevant advantage of this system that we wanted to exploit in order to identify yeast SDH operon products. This system is expected to have less non-specific background then the method that relies on utilizing antibodies. Additionally, this system can potentially be used to determine subunit stability in *E. coli* cells. This could be useful for distinguishing between rapid degradation of expressed subunits and very low expression levels.

As developed by Tabor and Richardson (137), the T7 RNA polymerase/promoter expression system maintains two plasmids within the same *E. coli* cell. Plasmid pGP1-2 contains the gene for the T7 RNA polymerase under the control of the λ p_L promoter that is repressed by a temperature-sensitive repressor (cI_{857}). The second maintained plasmid is the expression vector which contains the T7 promoter upstream of the gene of interest. Induction is achieved by shifting the growth temperature from 30 °C to 42 °C thereby inactivating the cI_{857} repressor. Alternatively, the strain BL21(DE3) carries the T7 RNA polymerase gene under the control of the *lacUV5* promoter as a lysogen in the *E. coli* chromosome and can be induced by the addition of IPTG (136). The procedure, as originally described, utilizes *E. coli* K38 and many researchers have found this strain particularily good for producing low backgrounds during ^{35}S-labeling. Therefore, we decided to use the two plasmid system in *E. coli* strain K38.

For use in this system, the synthetic yeast SDH operon was modified to place it under the control of the T7 RNA polymerase promoter creating plasmid pSdhOpT7. The construction of this plasmid was as described in Chapter 2. In this construct, the *frd* promoter-containing region (nucleotides 1-774 of the synthetic yeast SDH operon) was removed and the remaining operon (nucleotides 775-5090) was placed behind the T7 promoter provided by the vector pBC KS$^-$. The sequence extending from the T7 promoter through to 100 bp downstream of the *Sac* II restriction site (nt 828) of the yeast SDH operon in pSdhOpT7 was confirmed by sequence analysis. To identify the gene products, I used an ^{35}S-methionine/^{35}S-cysteine mixture, Trans^{35}-Label™, for radiolabeling. Following labeling, the proteins from whole cell lysates were concentrated by TCA precipitation from 200 µl of culture, separated on a 10% Tricine-SDS PAGE gel, and visualized by audoradiography. Labeling in *E. coli* harbouring pGP1-2 (K38/pGP1-2), both pGP1-2 and the empty expression vector pBC KS$^-$ (K38/pGP1-2/pBC KS$^-$), or both pGP1-2 and pSdhOpT7 (K38/pGP1-2/pSdhOpT7) was examined. We expected that K38/pGP1-2/pSdhOpT7 would produce four labelled polypeptides migrating with apparent molecular weights corresponding to the sizes of the four mature subunits of yeast SDH (i.e. 67-kDa, 28-kDa, 14-kDa, and 17-kDa, respectively). Results presented in Figure IV-7, lane 1, however, show labeling of at least nine polypeptides in K38/pGP1-2/pSdhOpT7 lysates (labelled bands i-ix in order of decreasing apparent size). Three of the bands can be immediately eliminated as products of the yeast SDH operon genes due to their presence in the negative controls. One, the upper band migrating near the 104-kDa molecular weight marker, named band i, is seen in all three cell lysates and is likely the T7 RNA polymerase which is a single polypeptide of 96-kDa. The second, a prominent band migrating near 28-kDa (band iv), is also present in the negative control containing the empty expression vector pBC KS$^-$ (K38/pGP1-2/pBC KS$^-$). This band is consistent with the expected size for chloramphenicol transferase (CAT), a 24.8-kDa protein, which is the selection marker on the pBC KS$^-$ vector. Expression and labeling of this protein is possible because the CAT gene is oriented in the same direction as the T7 promoter in the pBC KS$^-$ plasmid. The T7 RNA polymerase enzyme is known to be highly processive and to terminate transcription less frequently then *E. coli* RNA polymerase allowing its transcription to circumnavigate a plasmid resulting in RNA several times the plasmid length in size. Thus, the CAT gene is expressed under the control of the T7 promoter in pBC KS$^-$ and pSdhOpT7 and is selectively labeled. A third band (band iii) seen migrating near 36-kDa is also present in the negative control, K38/pGP1-2, and may be the same protein as seen in K38/pGP1-2/pSdhOpT7. Band iii could be a background protein or the 37-kDa protein that is detected by Sdh3p-antiserum in Figures IV-3 and IV-6. That leaves six remaining bands as candidates for products of the yeast SDH operon genes.

Figure IV-7, lane 1 shows the identification of a 67-kDa polypeptide (band ii) produced by *E. coli* harbouring the yeast SDH operon construct. This species is not present in either negative control K38/pGP1-2/pBC or K38/pGp1-2. This band is consistent with the expected migration of Sdh1p and is the first evidence of Sdh1p being expressed and accumulated within the cell. The cellular location of this Sdh1p product can not be determined from this experiment.

There is a broad band migrating at 28-kDa near where Sdh2p is expected (band iv). Additionally, there is a doublet running near 19 kDA (bands vi, and vii). I believe the upper band of the doublet (band vi) is an Sdh2p degradation product (band iv) based on the observation that it was also detected as an immunoreactive species by Western blot analysis of both the membrane fraction of the 12 hr time point in the 72 hr time course (Figure IV-2, panel A, lane 5) and in Figure IV-9, lane 6 (to be presented below) using anti-Sdh2 antiserum.

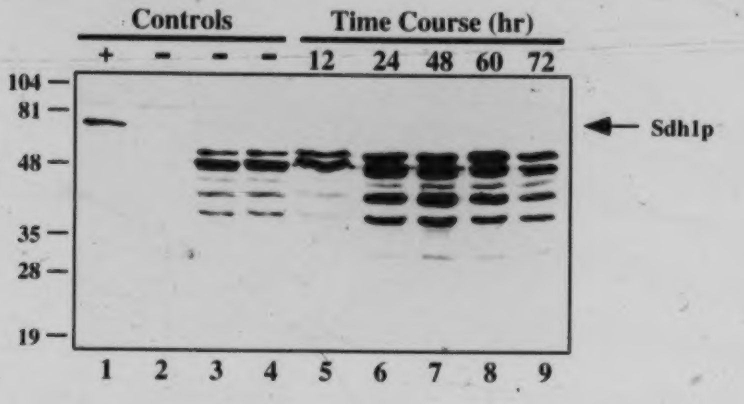
The anchor subunits, Sdh3p and Sdh4p, are more difficult to clearly identify in Figure IV-7. Sdh3p (16.7-kDa) and Sdh4p (16.6-kDa) are expected to migrate on a Tricine-SDS PAGE gel system with apparent molecular weights of 14-kDa and 17-kDa respectively (17). Band viii migrated as a broad species at a position corresponding to that expected for Sdh3p in K38/pGP1-2/pSdhOpT7 lysates. A narrower band viii is also present in the negative control K38/pGP1-2/pBC KS$^-$. The broad band viii in lane 1 may consist of a doublet consisting of the upper background protein and the lower species migrating at the expected size for Sdh3p. In the expression studies of the synthetic yeast SDH operon under *frd*-promoter control, an Sdh3p immunoreactive species was observed to be migrating at approximately 37 kDa. In this T7-promoter expression study, there is also a band near 37 kDa (band iii). However, there is also a band close to this size in the negative contol, K38/pGP1-2 as mentioned above. It is possible these are not the same polypeptides, and thus the labeled 37-kDa band could be a background band, or an aberrantly-migrating product of the *SDH3* gene.

Band vii migrates at 17-kDa near the position where Sdh4p is expected. Finally, the fastest migrating protein, band ix, is not observed in either of the negative controls. It does not correspond to any degradation products previously observed and can not be assigned.

The ^{35}S-labeling expression studies succesfully identified an Sdh1p product for the first time, and also clearly shows Sdh2p (bands ii and iv respectively). The other bands are more difficult to conclusively assign. It was decided to try to identify the unassigned bands by running non-radiolabelled samples on a second Tricine-SDS PAGE gel and performing Western blot analyses with the Sdh1p, Sdh2p, and Sdh3p antisera. No immunoreactive species were detected for the whole cell lysates (data not shown).

A larger scale expression study under T7 promoter control was carried out in the absence of radiolabelling to prepare cellular fractions which could be concentrated enough to be detected by antibodies. No rifampicin or Trans^{35} -Label™ were added to cells. Samples were separated into membrane, soluble, and cellular debris fractions for immunodetection following separation on a Tricine-SDS PAGE gel. Results are presented in Figures IV-8, IV-9, and IV-10. Strong immunoreactive bands were detected with the Sdh1p, Sdh2p, and Sdh3p sera. All subunits were detected solely in the cellular debris fractions of the *E. coli* harbouring the synthetic yeast SDH operon. The subunits are expected to be present in the whole cell fractions as well but, as previously observed, they are not abundant enough to be detected by antibodies. Sdh1p in the cellular debris fraction, migrates at the expected apparent molecular weight of 67-kDa (Figure IV-8, lane 8), corresponding to the migration seen in the positive control containing wildtype mitochondria. Sdh2p migrates at the expected 28-kDa (Figure IV-9, lane 6) corresponding to the positive control containing wildtype yeast mitochondria. There is also a prominent band migrating near 19-kDa that is not observed in the negative controls. This is likely an Sdh2p degradation product and has been detected previously (Figures IV-2 and IV-7). Sdh3p in Figure IV-10 migrates at the expected apparent molecular size of 14-kDa (lane 8) as seen for the yeast mitochondrial positive control (lane 1). There is no band migrating at the higher molecular weight of 37-kDa corresponding to the Sdh3p immunoreactive species detected previously in the membrane fraction of the 12 hr time point of expression under the *frd*-promoter (Figure IV-3, Panel A, lanes 5 and 9; Figure IV-6, lanes 8, 9, 10). These results suggest that under the T7 promoter, subunits are being expressed, but may be forming inclusion bodies within the *E. coli*.

A) Membrane Fractions



B) Soluble Fractions

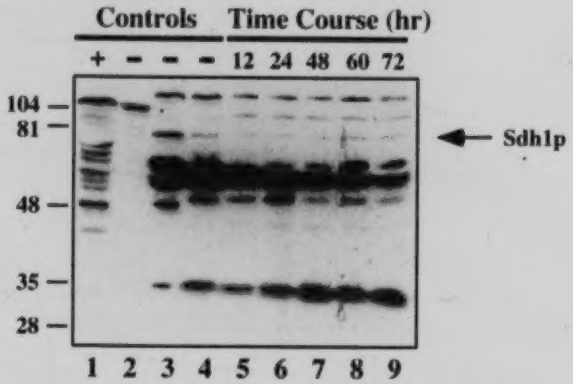
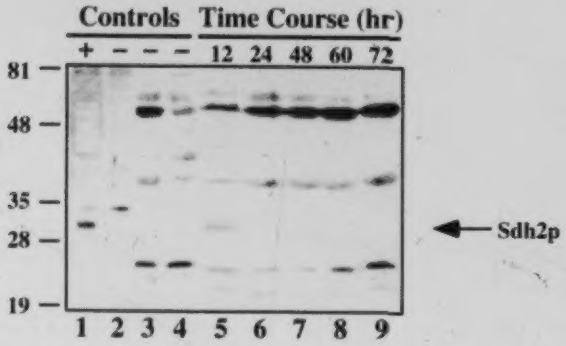


Figure IV-1. Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh1p Antiserum.

HB101 transformed with either pSdhOpF or pUC19e were induced for expression by anaerobic growth in glycerol-fumarate medium. Cells were harvested at various times after induction as indicated in hours above the gels. A: Membrane fractions. B: Soluble fractions. Equal amounts of protein (50 µg) were run on SDS-PAGE gels (A, 12%; B, 10%). Proteins were transferred to nitrocellulose and Western blots were performed using the *S. cerevisiae* Sdh1p antiserum. Arrows indicate the expected size of the subunit. Lanes 1-4, positive control (+), and negative controls (-). lane1, mitochondria isolated from wildtype *S. cerevisiae* (D273-10B); lane 2, mitochondria isolated from sdh1-knockout *S. cerevisiae* strain (Sdh1Ad1); lane 3, *E. coli* SDH-knockout strain (GVK124); lane 4, HB101 transformed with pUC19e harvested 48 hr after induction: Lanes 5-9, HB101 transformed with pSdhOpF harvested at various times after induction as indicated in hrs above the lanes. The positions of the size markers in kDa are indicated on the left.

A) Membrane Fractions



B) Soluble Fractions

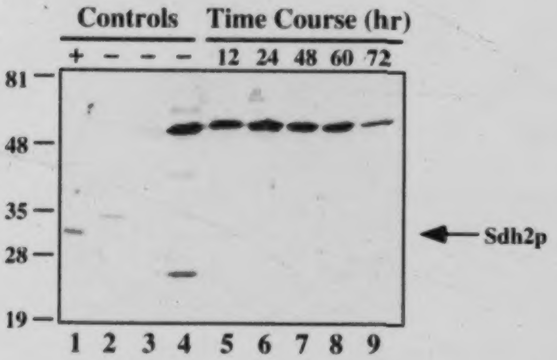
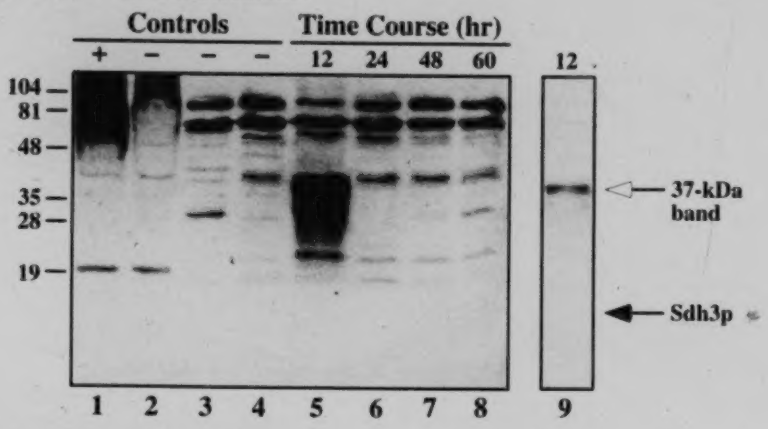


Figure IV-2.: Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh2p Antiserum.

HB101 transformed with either pSdhOpF or pUC19e were induced for expression by anaerobic growth in glycerol-fumarate medium. Cells were harvested at various times after induction as indicated in hours above the gels. A: Membrane fractions. B: Soluble fractions. Equal amounts of protein (50 μg) were run on 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose and Western blots were performed using the *S. cerevisiae* Sdh2p antiserum. The arrow indicates the expected size of the subunit. Lanes 1-4, positive control (+), and negative controls (-). lane1, mitochondria isolated from wildtype *S. cerevisiae* (D273-10B); lane 2, mitochondria isolated from sdh1-knockout *S. cerevisiae* strain (Sdh1Ad1); lane 3, *E. coli* SDH-knockout strain (GVK124); lane 4, HB101 transformed with pUC19e harvested 48 hr after induction: Lanes 5-9, HB101 transformed with pSdhOpF harvested at various times after induction as indicated in hrs above the lanes. The positions of the size markers in kDa are indicated on the left.

A) Membrane Fractions



B) Soluble Fractions

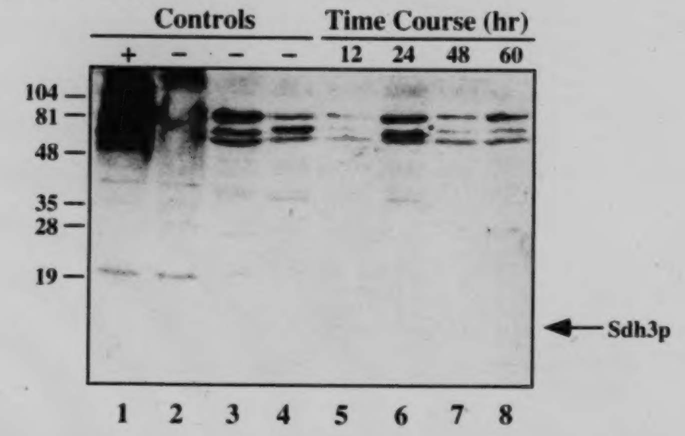


Figure IV-3. Western Blot Analysis of Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* from the *frd* Promoter over 72 hours Using Sdh3p Antiserum.

HB101 transformed with either pSdhOpF or pUC19e were induced for expression by anaerobic growth in glycerol-fumarate medium. Cells were harvested at various times after induction as indicated in hours above the gels. A: Membrane fractions. B: Soluble fractions. Equal amounts of protein (50 µg) were run on 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose and Western blots were performed using the *S. cerevisiae* Sdh3p antiserum. The solid arrows indicate the expected position of the Sdh3p subunit. The open arrow indicates the position of the 37-kDa immunoreactive band. Lanes 1-4, positive control (+), and negative controls (-). lane 1, mitochondria isolated from wildtype *S. cerevisiae* (D273-10B); lane 2, mitochondria isolated from sdh3-knockout *S. cerevisiae* strain (Sdh3W3); lane 3, *E. coli* SDH-knockout strain (GVK124); lane 4, HB101 transformed with pUC19e harvested 48 hr after induction: Lanes 5-8, HB101 transformed with pSdhOpF harvested at various times after induction as indicated in hrs above the lanes; lane 9 (Panel A), lane 5 exposed to film for a shorter time period. The positions of the size markers in kDa are indicated on the left.

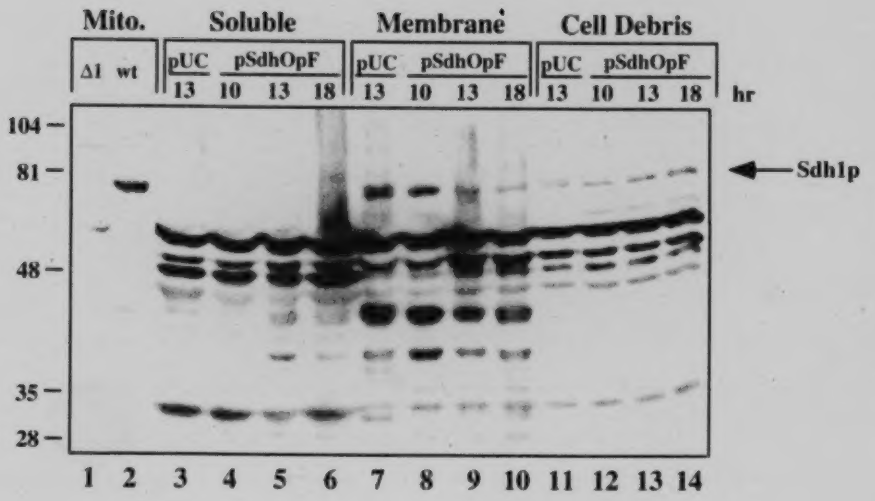


Figure IV-4. Western Blot Analysis Using the Sdh1p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli* .

HB101 transformed with either pSdhOpF or pUC19e (pUC) were grown anaerobically in glycerol-fumarate medium (lanes 3-14). Cells were harvested at various times following induction as indicated in hours above the gel, and fractionated. Equal amounts of protein (50 μg) were run on a 10 % SDS-PAGE gel. Proteins were transferred to nitrocellulose and a Western blot was performed using the *S. cerevisiae* Sdh1p antiserum. The arrow indicates the expected position of the Sdh1p subunit. lane 1, mitochondria isolated from sdh1-knockout *S. cerevisiae* strain, Sdh1Ad1 (Δ1); lane 2, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B (wt); lanes 3-6, HB101 soluble fractions; lanes 7-10, HB101 membrane fractions; lanes 11-14, HB101 cell debris fractions. The positions of the size markers in kDa are indicated on the left.

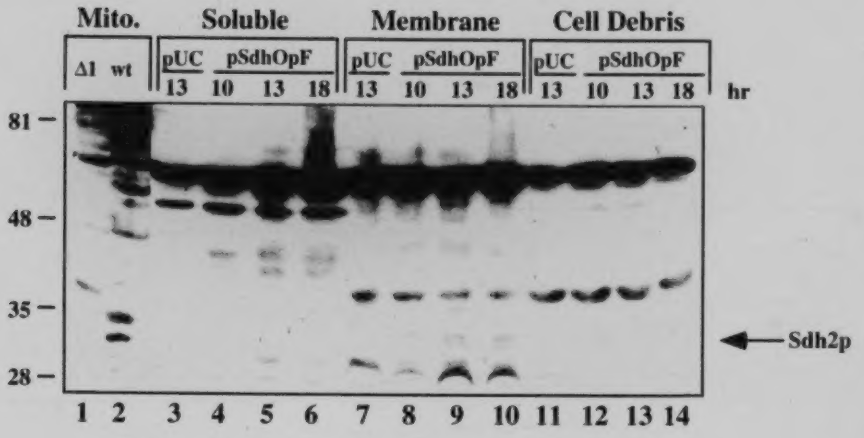


Figure IV-5. Western Blot Analysis Using the Sdh2p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli*.

HB101 transformed with either pSdhOpF or pUC19e (pUC) were grown anaerobically in glycerol-fumarate medium (lanes 3-14). Cells were harvested at various times following induction as indicated in hours above the gel, and fractionated. Equal amounts of protein (50 µg) were run on a 10 % SDS-PAGE gel. Proteins were transferred to nitrocellulose and a Western blot was performed using the *S. cerevisiae* Sdh2p antiserum. The arrow indicates the expected position of the Sdh2p subunit. lane 1, mitochondria isolated from sdh1-knockout *S. cerevisiae* strain, Sdh1Ad1 (Δ1); lane 2, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B (wt); lanes 3-6, HB101 soluble fractions; lanes 7-10, HB101 membrane fractions; lanes 11-14, HB101 cell debris fractions. The positions of the size markers in kDa are indicated on the left.

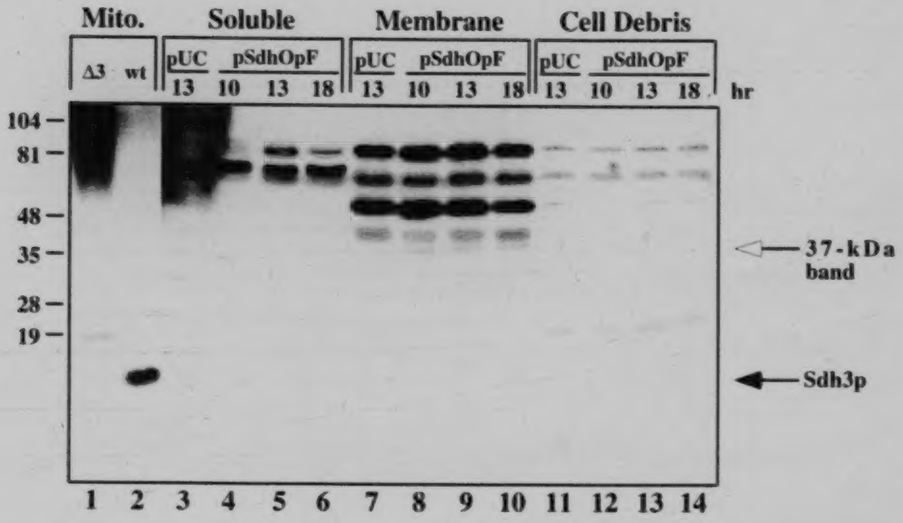


Figure IV-6. Western Blot Analysis Using the Sdh3p Antiserum of the Anaerobic Expression of the Synthetic Yeast SDH Operon in *E. coli*.

HB101 transformed with either pSdhOpF or pUC19e (pUC) were grown anaerobically in glycerol-fumarate medium (lanes 3-14). Cells were harvested at various times following induction as indicated in hours above the gel, and fractionated. Equal amounts of protein (50 μg) were run on a 10 % Tricine-SDS PAGE gel. Proteins were transferred to a PVDF membrane and a Western blot was performed using the *S. cerevisiae* Sdh3p antiserum. The solid arrow indicates the expected position of the Sdh3p subunit. The open arrow indicates the position of the 37-kDa immunoreactive band. lane 1, mitochondria isolated from sdh3-knockout *S. cerevisiae* strain, Sdh3W3 (Δ3); lane 2, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B (wt); lanes 3-6, HB101 soluble fractions; lanes 7-10, HB101 membrane fractions; lanes 11-14, HB101 cell debris fractions. The positions of the size markers in kDa are indicated on the left.

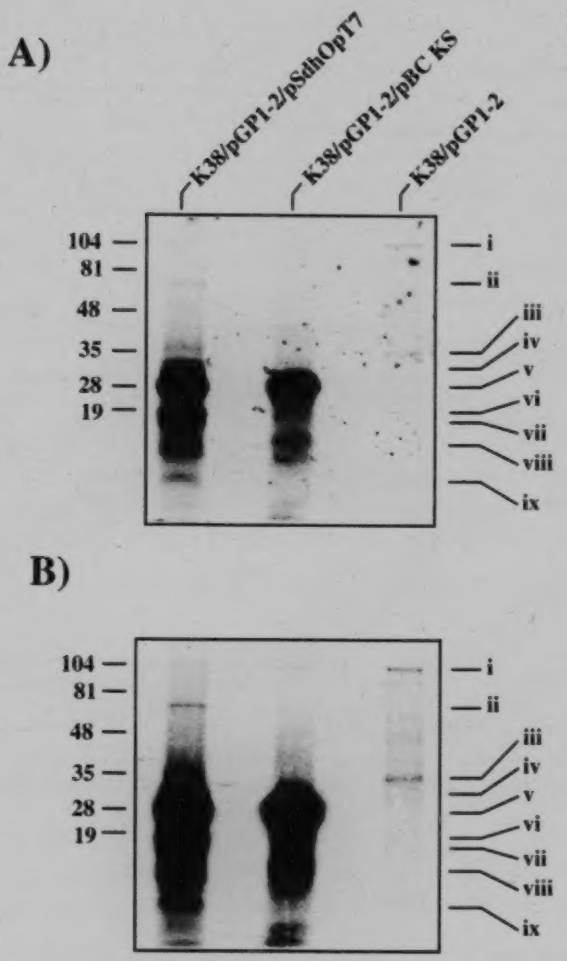


Figure IV-7. Autoradiogram of Aerobic Expression of the Synthetic Yeast SDH Operon From The T7 Promoter

E. coli strain K38/pGP1-2 either alone, or transformed with either pBC KS$^-$ or pSdhOpT7, were grown aerobically at 30 °C and induced for both expression and specific ^{35}S-methionine/cysteine labelling of plasmid-encoded proteins by a shift to 42 °C. Whole cell lysates were prepared from 200 µl of culture, subjected to separation on a 10 % Tricine-SDS PAGE gel and autoradiographed. Panel A: 5 hr exposure to film. Panel B: 24 hr exposure to film. Nine major proteins (i-ix) were radiolabelled as indicated on the right. The positions of size markers in kDa are indicated on the left.

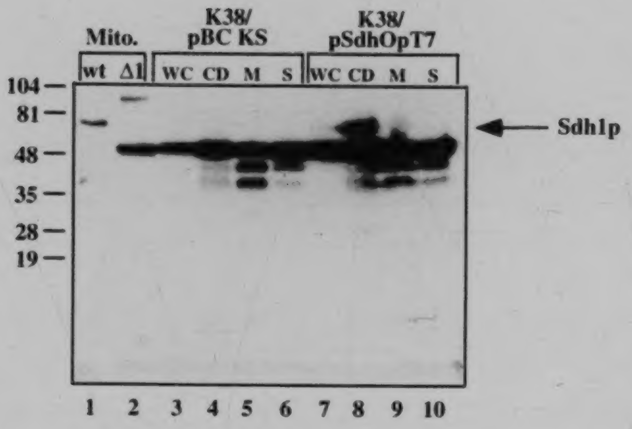


Figure IV-8. Western Blot Analysis Using Sdh1p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter

E. coli strain K38/pGP1-2 was transformed with the empty expression vector (pBC KS-, lanes 3-6), or the synthetic yeast SDH operon-containing plasmid (pSdhOpT7, lanes 7-10) and induced for expression by growth at 42 °C. Cells were harvested 2 hrs after induction and whole cell (WC), cell debris (CD), membrane (M), and soluble (S) cellular fractions were obtained. Fifty μg of protein were separated on a 10% Tricine-SDS PAGE gel and transferred to a PVDF membrane. A Western blot was performed using the *S. cerevisiae* Sdh1p antiserum. The arrow indicates the expected size of the Sdh1p subunit. Lane 1, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B(wt); Lane 2, mitochondria isolated from *sdh1*-knockout *S. cerevisiae* strain, Sdh1Ad1 (Δ1). The positions of the size markers in kDa are indicated on the left.

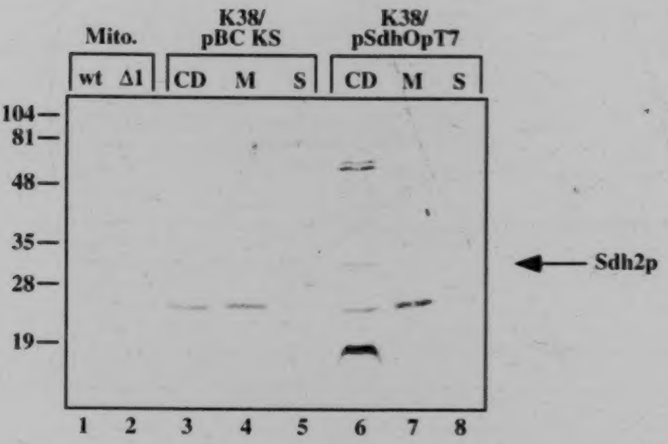


Figure IV-9. Western Blot Analysis Using Sdh2p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter

E. coli strain K38/pGP1-2 was transformed with the empty expression vector (pBC KS-), or the synthetic yeast SDH operon-containing plasmid (pSdhOpT7) and induced for expression by growth at 42 °C. Cells were harvested 2 hrs after induction and cell debris (CD), membrane (M), and soluble (S) cellular fractions were obtained (lanes 3-8) . Fifty μg of protein were separated on a 12 % -SDS PAGE gel and transferred to a nitrocellulose membrane. A Western blot was performed using the *S. cerevisiae* Sdh2p antiserum. The arrow indicates the expected size of the Sdh2p subunit. Lane 1, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B(wt); Lane 2, mitochondria isolated from *sdh1*-knockout *S. cerevisiae* strain, Sdh1Ad1 (Δ1). The positions of the size markers in kDa are indicated on the left.

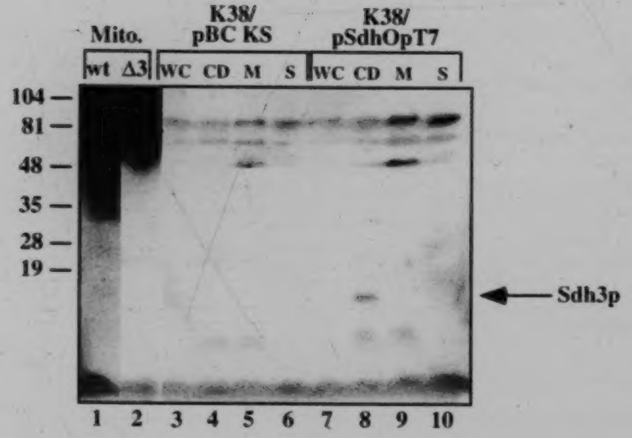


Figure IV-10. Western Blot Analysis Using Sdh3p Antiserum of Aerobic Expression of the Synthetic Yeast SDH Operon From the T7 Promoter

E. coli strain K38/pGP1-2 was transformed with the empty expression vector (pBC KS-, lanes 3-6), or the synthetic yeast SDH operon-containing plasmid (pSdhOpT7, lanes 7-10) and induced for expression by growth at 42 °C. Cells were harvested 2 hrs after induction and whole cell (WC), cell debris (CD), membrane (M), and soluble (S) cellular fractions were obtained. Fifty µg of protein were separated on a 10% Tricine-SDS PAGE gel and transferred to a PVDF membrane. A Western blot was performed using the *S. cerevisiae* Sdh3p antiserum. The arrow indicates the expected size of the Sdh3p subunit. Lane 1, mitochondria isolated from wildtype *S. cerevisiae*, D273-10B(wt); Lane 2, mitochondria isolated from *sdh3*-knockout *S. cerevisiae* strain, Sdh3W3 (Δ3). The positions of the size markers in kDa are indicated on the left.

Chapter V: Discussion

V-1. Discussion

The work presented in this thesis describes the bacterial expression of the yeast mitochondrial succinate dehydrogenase (SDH) enzyme complex. SDH is an excellent model system for studies regarding structure, function, and biogenesis of mitochondrial membrane-bound multisubunit enzyme complexes. It is essential for oxidative phosphorylation and is unique in that it is a member of both the citric acid cycle and the membrane-associated respiratory chain. Of the oxidative phosphorylation enzyme complexes, SDH is the simplest, being composed of only four polypeptides. Also, SDH has the same general composition in procaryotes and eucaryotes. In contrast, the eucaryotic Complexes I, III, and IV have additional subunits compared to their procaryotic counterparts.

The SDH enzymes of bovine heart mitochondria, *E. coli*, *B. subtilis*, and *S. cerevisiae* and the FRD enzymes of *E. coli* have been extensively studied. The research within the Lemire laboratory has focused on the SDH complex of *S. cerevisiae*. Yeast offers a number of significant genetic advantages as a model system such as short generation times and the ability to study respiration-deficient mutants. However, yeast as a model system would benefit from the ability to generate large quantities of purified native protein for biophysical and mutational studies. Our laboratory has already attempted to overproduce SDH in yeast (31, 112). Although the subunits were overproduced, functional, assembled enzyme was not overproduced. Therefore, we decided to overexpress the yeast SDH in *E. coli*.

The experimental design and rationale for the approach to express the yeast SDH in *E. coli* are based on the previous success of others in overproducing the closely related *E. coli* FRD enzyme. The *E. coli* FRD complex has been successfully overproduced thirty to forty fold in *E. coli* by cloning the *frd* operon into a multicopy plasmid and inducing expression under anaerobic conditions (86). The strategy consisted of creating a synthetic yeast SDH operon based on the architecture of the *frd* operon. FRD is structurally similar to SDH, and since *E. coli* is able to insert the FAD cofactor and iron-sulfur clusters in large amounts of FRD, it was hoped the same would be true of the yeast SDH.

I have examined the expression of the synthetic yeast SDH operon under the control of the *E. coli frd* operon under anaerobic growth conditions and under the control of the bacteriophage T7 promoter under aerobic conditions. Identification of the yeast polypeptides expressed from the *E. coli frd* promoter relied on detection of the the yeast Sdh1p, Sdh2p, and Sdh3p subunits with subunit-specific antisera following separation by SDS-PAGE electrophoresis. No antiserum against the yeast Sdh4p is available. Yeast polypeptides expressed from the T7 promoter were additionally identified by radiolabeling.

The anaerobic expression of the yeast SDH failed to produce a high enough level enzyme for use in further studies. The *frd*-promoter driven expression of the yeast SDH operon was tested in the *E. coli* strain, HB101, over a 72 hr time course following anaerobic induction. Immunoblots of *E. coli* membranes using anti-Sdh2p antiserum showed a low accumulation of an immunoreactive protein with an apparent molecular weight consistent with that of the yeast Sdh2p. Antisera against Sdh1p did not detect any immunoreactive bands of the expected size, while the Sdh3p antiserum detected a protein migrating with an unexpected size (i.e. 37 kDa instead of the expected 14 kDa). The *frd* promoter was expected to direct constant expression of the synthetic yeast SDH operon under anaerobic conditions. Instead, accumulation of the expressed yeast subunits displayed a temporal pattern. Products were detected in equal amounts in the 12 and 18 hr membrane fractions. No protein products were detected 24 hr following induction. These data indicate that either anaerobic expression of the yeast SDH mature subunits under the *frd*-promoter was low or that the subunits were unstable.

Of the three available antisera, only the anti-Sdh2p antiserum detected a protein product of the expected size. An Sdh3p immunoreactive species was detected, but migrated with an apparent molecular weight of 37 kDa instead of the expected 14 kDa. The reason for this is not known. A similar observation has been reported (15). This group reported that during homologous overexpression of the NADH dehydrogenase fragment of Complex I from *E. coli*, expression of two of the six genes, *nuoC* and *nuoD*, led to the formation of only one protein, NuoCD. The authors suggested one possible explanation was that the TGA stop codon of *nuoC* in the *nuo*-operon may code for the natural amino acid selenocysteine, and noted that translation of TGA as selenocysteine would not change the reading frame of *nuoD* but lead to the translation of three more amino acids in NuoCD. An apparent molecular weight of 37 kDa is close to that which would be predicted for an Sdh3p-Sdh4p fusion protein. However, the *SDH3* stop codon in the yeast SDH operon is TAA, which does not code for selenocysteine. Furthermore, translation through the intergenic region between *SDH3* and *SDH4* would lead to a frame shift of the *SDH4* open reading frame resulting in a nonsense polypeptide. We do not believe the aberrantly migrating Sdh3p immunoreactive protein represents an Sdh3p-Sdh4p species. Conclusive identification of this 37 kDa species as Sdh3p will require further investigation.

Many investigators have successfully used the T7 polymerase/promoter expression system to overexpress proteins. The T7 system was considered an appropriate tool for testing for the expression of the yeast Sdh1p and examining expression over very short time periods. The *frd*-promoter was replaced with the bacteriophage promoter, thus placing the expression of the SDH operon under control of the T7 promoter. Protein products were identified using ^{35}S-methionine/cysteine radiolabeling. Radiolabeling clearly identified a protein band of the expected size for the yeast Sdh1p. A band corresponding to Sdh2p was also identified. Although the background radiolabeling was low, positive identification of Sdh3p and Sdh4p was more difficult. Bands of the expected size for the anchor subunits were present but the possibilty remains that they are degradation products of other plasmid-encoded proteins. The signal for Sdh1p was quite low. The four yeast SDH subunits have the following number of methionine/cysteine residues based on their predicted sequences: Sdh1p 26; Sdh2p, 22; Sdh3p, 3; Sdh4p, 9. These numbers include the initiator methionine, which is often removed following translation. The signal strengths for an equimolar ratio of subunits Sdh1p:Sdh2p:Sdh3p:Sdh4p would be approximately 9:7:1:3 respectively. Thus, Sdh1p is being expressed and accumulated, but to a lower degree than the other yeast subunits. The T7 promoter driven expression was repeated in the absence of radiolabeling for preparing cellular fractions. Expression of the synthetic yeast SDH operon under the control of the T7 promoter led to the production of three protein bands that are reactive with Sdh1p, Sdh2p, and Sdh3p antisera and migrated on an SDS-Page gel at the expected positions for the three yeast SDH subunits, respectively. Surprisingly, these yeast subunits, were all located in the cell debris fraction. The collection of the cell debris fraction by centrifugation at 8 000 x *g* is too low to pellet membranes (134) and may indicate that the yeast SDH subunits are accumulating within the host cell as an inclusion body (153).

The goal of this work was to produce functional assembled yeast SDH enzyme in bacteria. Under the *frd* promoter, however, only low levels of the yeast Sdh2p subunit were detected. The T7 promoter directed expression of Sdh1p, Sdh2p, and Sdh3p subunits, but they are apparently present in an insoluble form. Overexpression of a multisubunit, cofactor-containing, membrane bound eucaryotic enzyme complex in bacteria is a challenging goal. In general, some of the known reasons that proteins fail to be expressed in *E. coli* include: 1) Cell toxicity of the recombinant protein resulting in the interference of gene expression, or a compromise of cell integrity(136), 2) Inefficient translation due to factors such as major differences between the codon usage of the recombinant protein and the host *E. coli* codon usage (44) and 3) Instability of the expressed protein due to rapid degradation by host proteases (5). These three possibilities will now be discussed.

The possibilities that the mRNA or the translated proteins from the synthetic yeast SDH operon are toxic to the host cell seem unlikely as cells harbouring the expression plasmid grew as well as cells containing a control plasmid. However, during anaerobic expression, cells stopped accumulating Sdh2p by 24 hr following induction, which may indicate a mild toxicity of the expressed recombinant protein (137).

Translation of a foreign message can be limited in *E. coli* because of differences in codon usage between procaryotes and eucaryotes (44, 74). The genetic code is highly degenerate in that it contains 64 possible triplet codons to specify only 20 amino acids and 3 termination codons. Generally, however, the alternative synonymous codons are not used with equal frequencies (62). This codon bias is species specific (41). *S. cerevisiae* has a strong bias for 25 of the 61 possible coding triplets, while *E. coli* has a bias for 22 of the 61 codons (8). In at least four instances (Ala, Arg, Leu, Val) the preferred *E. coli* codons are incompatible with the preferred yeast codons (8, 63). The preferred codons are those that correspond to the most abundant isoaccepting tRNA molecules (40, 62). The differences between the choices of synonymous codons in yeast and *E. coli* are attributed to the differences in the actual populations of isoaccepting tRNAs present in the two organisms (61). Within a species, the degree with which the preferred codons occur in a given gene is strongly correlated with the gene's level of expression (40). It has been proposed that this permits the mRNAs of proteins that are required in high abundance to be rapidly and smoothly translated (8). Conversely, genes that contain a substantial number of codons that are rarely used in *E. coli* may be expressed inefficiently (63).

There is experimental evidence that the abundant presence of low-usage *E. coli* codons, such as AGG/AGA (specifying Arg), can result in a reduced level of gene expression in *E. coli* (16, 114). Brinkmann *et al.* showed that the expression of three particular proteins each with a high content of rare Arg codons in their respective genes was limited in *E.coli* (16). High-level production of these proteins (>30% of total cellular protein) was achieved by supplementing the expression host with the tRNA$^{(AGA/AGG\ (Arg))}$ by coexpressing the *dna Y* gene. The *E. coli dna Y* gene encodes the minor arginine tRNA$^{(AGA/AGG\ (Arg))}$ (36). The authors compared expression of 8 heterologous proteins in *E. coli* and found that the 3 genes containing 3% or more of rare Arg codons in their genes (human tissue-type plasminogen activator, human pro-urokinase, and gp41 of human immunodeficiency virus) required the concomitant overexpression of the *dna Y* gene for high-level expression (16). The level of expression was increased from less than 5% of total cellular protein to 30% of total cellular protein in the presence of the overexpressed *dna Y* gene product. The authors found that the *dna Y* gene product was not only important in increasing translation, but also seemed to play an important role in replication. They suggested that codon usage in general may not be a limiting factor in recombinant gene expression, but rather that the *dna Y* gene product improves recombinant protein yield of certain proteins by both supplementing the minor tRNA pool and improving chromosomal and plasmid replication. Similar studies have shown that the approach of co-overexpressing the *dna Y* gene often has inconsistent results, and many of the determining factors of this strategy are not yet understood (49).

The synthetic yeast SDH operon encodes 4 polypeptides with a total of 1165 amino acids. Of these residues, there are 46 (3.9%) Arg encoded by the rare AGA/AGG codons. Sdh1p contains 29 low-usage Arg codons (4.7%), Sdh2p contains 11 (4.4%), Sdh3p contains 4 (2.7%), and Sdh4p contains 2 (1.3%) low-usage Arg codons. Thus Sdh1p and Sdh2p have a particularly high content of rare Arg codons and may be translationally limited in *E. coli*. The coexpression of the *dna Y* gene and the synthetic yeast SDH operon may lead to improved expression levels of assembled yeast SDH in *E. coli*.

The base composition of the first 10 codons can be especially important for efficient expression (74), while the codon usage for the rest of the gene is less important (102). The expression of many heterologous proteins such as bacteriorhodopsin (74), and cytochrome p450 17 α-hydroxylase (6) were optimized for high-level production by altering codons within the first 10 codons to match the preferred codon choices of *E. coli*. To optimize expression of the synthetic yeast SDH operon in *E. coli*, any low-usage *E. coli* codons within the first 10 codons of each gene were altered to preferred codons. It is impractical to alter the remaining low-usage Arg codons as they are distributed throughout the yeast SDH operon.

Eucaryotic membrane proteins tend to be particularly difficult to express in *E. coli* (129). Using various constructs of a gene for a liver mitochondrial proton-phosphate symporter, a study addressing the specific problems of expressing eucaryotic membrane-proteins in bacteria, identified a problem at the level of translation (33). In addition to problems at the level of transcription, and gene product toxicity, the authors ruled out low-codon usage as a limiting factor. The authors ascribed the failure to express the eucaryotic membrane protein in *E. coli* to a translation problem that involved an unknown incompatibility of the mRNA with *E. coli* ribosomes. In contrast, Kitjima-Ihara (76) reported the functional overexpression of a yeast mitochondrial membrane protein, the rotenone-insensitive internal NADH-quinone oxidoreductase, in *E. coli*. This demonstrates that a yeast gene encoding a mitochondrial membrane protein can be successfully expressed in *E. coli*.

The translation initiation efficiency in *E. coli* may also be influenced by the initiation codon. *FrdA* belongs to a small group of genes in *E. coli* that use a GTG initiation codon instead of the more common ATG (25). Because this feature has been so highly conserved in *Enterobacteriaceae frd* operons (26), it was decided to use the GTG initiation codon for the *SDH1* gene of the synthetic yeast SDH operon. Reddy *et al.* (107) have shown that a

GTG codon is up to threefold less efficient than an ATG codon in initiating translation in *E. coli*. In agreement with these findings, Jones and Gunsalus (71) reported that expression of a fusion protein, frdA'-*lacZ*$^+$, initiating with an ATG codon gave a five-fold higher level than the same protein fusion initiating with a GTG codon. Particularly relevant is the recent finding of Maklashina *et al.* (93) who anaerobically overexpressed the *E. coli sdh* operon by placing it under the control of the *E. coli frd* promoter. The first gene in the *E. coli sdh* operon, *sdhC*, starts with an ATG codon in contrast to the *frdA* GTG codon in the *frd* operon. Synthetic *E. coli sdh* operon constructs were made with either ATG or GTG as the start codon (93). It was found that the level of enzyme present in the membrane produced from the ATG-construct was about 30% higher than that from the GTG-construct. However, growth was inhibited by the ATG construct (93). Based on these observations, the expression level of the synthetic yeast SDH operon may be improved by replacing the GTG initiation codon of the mature *SDH1* gene with ATG.

An efficient translation rate in itself is not necessarily sufficient for producing high levels of recombinant protein. Many overproduced proteins fail to form their correct three-dimensional structure and become susceptible to either degradation or accumulation as insoluble inclusion bodies. This phenomenon constitutes the third general reason that proteins fail to express in *E. coli* (5).

Some heterologous proteins are susceptible to degradation by host cell proteases. Proteolysis is a selective, regulated process that is involved with metabolic activities such as the removal of abnormal and incorrectly folded proteins. Some of the structural determinants of protein instability have been defined. The 'N-end rule' proteolytic pathway is functional in *E. coli* and determines a protein's stability (138). Proteins with the amino acids; Arg, Lys, Phe, Leu, Trp, and Tyr are targeted for 'N-end rule' proteolysis and have half lives of only 2 min while all other amino acids situated at the amino terminus of the same polypeptide confer half-lives of > 10 hr (138). Following translation in *E. coli*, the N-terminal fMet is often cleaved. If one of the six 'N-end rule' amino acids is then exposed, the polypeptide will be targeted for rapid degradation. The second amino acid of the polypeptide determines the degree of removal of the N-terminal fMet. The extent of removal, as catalyzed by methionyl aminopeptidase, decreases as the size side chain of the second amino acid increases (59, 83). Little or no processing was observed when the following amino acids occupied the second position: His, Gln, Phe, Met, Lys, Tyr, Trp, Arg (59, 83). Therefore, proteins with Leu in the second position are likely to be unstable, since they would be targeted for rapid degradation following the removal of the fMet. In the synthetic yeast SDH operon, none of the four polypeptides contain an amino acid in the second position that would target them for degradation by the 'N-end rule' proteolytic pathway. The second amino acid of yeast Sdh4p was changed from Leu-32 to Ile-32 in order to create a restriction site for cassette-cloning. This change might also protect Sdh4p from being targeted for proteolytic degradation in *E. coli.*

Incorrectly folded proteins can also be susceptible to degradation. In yeast, Sdh1p and Sdh2p require assembly for stability, otherwise they are rapidly degraded. In contrast *Bacillus subtilis*, will accumulate the iron-sulfur and flavoprotein subunits when expressed alone (55).

The overexpression of recombinant proteins in *E. coli* can often result in the formation of inclusion bodies (73). Inclusion bodies are insoluble, extremely dense aggregates of recombinant protein. They are formed from the accumulation of folding intermediates rather than from the native or unfolded polypeptides and have no biological activity (42). The production of recombinant proteins as inclusion bodies has advantages including protection from proteolysis and the easy isolation of a high yield of recombinant protein (49). A major disadvantage, however, is the need to renature the recovered protein into a biologically active molecule. Active proteins can be recovered from inclusion bodies but it is very difficult to work out the necessary conditions (42). It is not possible to generalize or predict which proteins will be produced as inclusion bodies (42). Despite extensive studies on inclusion bodies, the mechanisms of their formation are still obscure. The general view at this time is that the nature of the expressed protein, the rate of its expression, and the level of its expression can all influence the formation of inclusion bodies (99). A high rate of expression may result in providing insufficient time for proper folding. A high local concentration in the cytoplasm, especially of hydrophobic proteins, may lead to precipitation. In addition, accessory folding proteins may not be adequately available for proper assembly. Growth temperature is also a major factor in dictating the formation of inclusion bodies. A lower temperature (20-30 °C) may result in an active soluble protein instead of an aggregate (128).

Strategies that have been developed to favour the formation of the native three-dimensional protein structure include; making amino acid substitutions that suppress aggregation, using highly soluble polypeptides as fusion partners to prevent the aggregation of the hybrid, co-overexpressing the native or heterologous folding accessory proteins with the target protein to assist proper assembly, creating a less reducing cytoplasmic environment to facilitate disulfide bond formation, reducing the rate of target protein synthesis, using a protease-deficient strain, and optimizing the growth conditions of the bacterial culture (38, 49).

The approach of engineering specific amino acid substitutions to prevent self-association has been demonstrated for a number of proteins (150). However, it is not always possible to predict which amino acid changes will provide the desired results, and more importantly it then becomes necessary to characterize the effect of the mutation on the function, stability, or structure of the mutated protein (38).

Fusion partners offer several advantages for facilitating high level expression and purification of recombinant proteins in *E. coli* (49). They can aid in preventing inclusion body formation, improve folding characteristics, decrease susceptibility to proteolysis, and provide the option of routine protein-purification schemes. Several proteins such as glutathione-S-transferase, maltose-binding protein, and thioredoxin have been developed as fusion partners (49). Neither of these two approaches are attractive strategies for improving expression of the yeast SDH operon as they involve making undesirable changes to the yeast subunits.

Coexpressing accessory folding proteins has been shown in certain cases to increase the level of production of several monomeric and multimeric proteins (38). Two diverse classes of accessory proteins exist in the cell to prevent the misfolding of newly synthesized polypeptides. Chaperones and foldases belong to this category. An important multicomponent chaperone complex consists of the GroEL (60 kDa) and the smaller GroES (10 kDa) proteins (GroESL). GroEL forms a characteristic doublet of heptameric rings which associates with one or two heptameric rings of GroES. This GroESL complex has a very broad substrate specificity and prevents misfolding by providing an environment for folding intermediates to attain their native conformation without forming inappropriate associations. The co-overexpression of homologous or heterologous folding helper proteins has been effective in facilitating assembly of several monomeric and multimeric heterologous expressed proteins in *E. coli* (38). The first demonstration that the assembly of foreign dimeric, octameric, and hexadecameric Rubiscos (ribulose bisphosphate carboxylases) in *E. coli* required GroESL was in 1989 (39). Since then at least 30 reports have been published of proteins with improved production in *E. coli* in the presence of the overexpressed GroESL chaperone complex (38). A dramatic example is the overexpression of rat neuronal nitric oxide synthase (nNOS) (115). nNOS is a complex 160 kDa polypeptide containing protoporphyrin IX heme, FAD, FMN and tetrahydrobiopterin as prosthetic groups. The coexpression of GroESL and nNOS genes in *E. coli* led to the production of > 20 mg/L assembled recombinant protein (115). The production of large amounts of recombinant nNOS also required the optimization of growth conditions in addition to coexpressing the molecular chaperone. A protease-deficient *E. coli* strain and the supplementation of the media with heme and flavin precursors were also required (115). For any given heterologous protein, only the chaperone that beneficially interacts with the folding intermediates will be effective in preventing misfolding.

Unfortunately, this correct substrate-chaperone match has to be found by trial and error. Other than GroESL and DnaK/J chaperone complexes, little is known about manipulating other chaperones (38). In some cases, chaperones cloned from the same source as the desired protein are necessary (23). Expression of the yeast SDH in *E. coli* may be improved by the co-expression of molecular chaperones.

The failure to accumulate yeast SDH in *E. coli* may reflect a high rate of polypeptide degradation within the cell. We had hoped to use the T7 polymerase/promoter expression system to distinguish between low expression and rapid degradation of the yeast SDH subunits. However, expression under the T7 promoter likely led to the formation of inclusion bodies which are resistant to proteolysis; these are not useful for determining the turnover rate of the recombinant polypeptides. The T7 promoter is known to be very strong and may be resulting in a rate of expression too rapid for proper folding of the yeast polypeptides. Lowering the growth temperature to 20 °C may prevent the formation of inclusion bodies, however, it is felt that the T7 promoter is probably not an appropriate choice of promoters for optimizing expression of the synthetic yeast SDH operon.

E. coli is the most frequently used procaryotic expression system for the high-level production of both procaryotic and eucaryotic proteins (49). However, despite its many advantages, the efficient expression of different genes is not routine. The overproduction of membrane proteins, as opposed to soluble proteins, has so far proven difficult and many of the obstacles remain obscure. Exceptionally high levels of expression have been achieved in some cases. The homologous overexpression of ATP synthase (144), fumarate reductase (147), and glycerol phosphate acyl transferase (152) all produced large quantities of functional protein that was inserted into tubular structures inside the *E. coli* host cell.

The heterologous expression of membrane proteins has been especially difficult and has only been successful in some exceptional cases (129). Bacteriorhodopsin from the archaebacterial *Halobacterium halobium* (74), and the rotenone-insensitive internal NADH-quinone oxidoreductase of yeast mitochondria (76) represent two relevant examples. Bacteriorhodopsin from *H. halobium* was one of the first heterologous membrane proteins to be successfully expressed in *E. coli*. This normally highly expressed protein was produced in a non-native form that could be refolded following reconstitution to produce enough properly-folded protein for biochemical studies (74). The level of expression in *E. coli* was improved by engineering the first 10 codons to correlate with preferred *E. coli* codons (102). The rotenone-insensitive internal NADH-quinone oxidoreductase sets a precedence for the successful functional expression of a yeast mitochondrial membrane protein in its native conformation (76). This protein consists of a single subunit with a non-covalently bound FAD, and no iron-sulfur clusters (76).

Of particular relevance to the goal of this thesis is the recent report of the homologous anaerobic overproduction of the *E. coli sdh* operon under the *E. coli frd*-operon promoter (93). The investigators were interested in demonstrating whether or not SDH could functionally replace FRD, and thus designed a construct to direct expression of the *E. coli* SDH under anaerobic conditions by utilizing the *frd-operon* promoter. Thus, they employed the same expression strategy we used to achieve similar goals. The study contains a number of observations with implications on this work. Firstly, the strategy to overexpress the aerobic-favoured enzyme in an anaerobic environment is clearly possible. In *E. coli,* the expression of SDH and FRD is tightly regulated, in part by the presence of oxygen. SDH is expressed maximally under aerobic conditions and tightly repressed under anaerobic conditions, while the opposite is true for FRD. Maklashina's work confirms that the anaerobic expression of a tightly regulated aerobic enzyme is not inherently deleterious (93).

Also interesting is the finding that the anaerobic overproduction of *E. coli* SDH under the *frd*-promoter leads to a 2-3 fold greater level of overproduction than the overexpression of *E. coli* SDH from its native promoter under aerobic conditions (93). This finding supports the choice of the *frd*-promoter and anaerobic growth conditions for driving the expression of the synthetic yeast SDH operon. The successful overexpression of the *E. coli* SDH and FRD lends support to the prospect of successful expression of the yeast SDH in *E. coli.*

V-2. Future Studies

In summary, the expression level of the yeast SDH produced in *E. coli* was insufficient for proceeding with further biochemical studies. Several approaches to improve the production of the synthetic yeast SDH operon should be tried.

To improve the translation efficiency of the synthetic yeast SDH operon in *E. coli*, there are two options worth exploring. First, genetically changing the GTG initiation codon of the *SDH1* gene to ATG may lead to higher levels of Sdh1p being produced. The other three yeast SDH genes all initiate with ATG. Second, codon usage is known to present a potential impediment to high-level gene expression in *E. coli* (63). Coexpressing the *E. coli* gene for the rare tRNA $^{(AGA/AGG)}$, *dna Y*, may help to minimize the effects of the presence of low-usage codons present in the synthetic yeast SDH operon. Despite the removal of rare codons from the first 10 codons of each of the four structural genes, there is still a large occurrence of low-usage Arg codons in the yeast SDH operon. Sdh1p and

Sdh2p of the synthetic yeast SDH operon each have a high number of low-usage Arg codons in their respective genes. While the success of this approach has been inconsistent, it may prove to be valuable in improving expression of the yeast SDH operon in *E. coli*.

Misfolding is a particularly vexing problem in the expression of eucaryotic proteins, especially those that are composed of multiple subunits, or that contain prosthetic groups. Misfolded proteins lead to lower yields of accumulated functional proteins following degradation by the cellular proteolytic machinery or accumulation in an insoluble form. There is a great deal of interest at this time in improving the usefulness of *E. coli* as a tool for gene expression and many strategies have been developed to improve the proper folding and assembly of recombinant overproduced proteins in *E. coli* . The approaches that are the most likely, and the most practical, to have a beneficial effect on the assembly of yeast SDH include the following: the coexpression of molecular chaperones, experimenting with alternative expression host strains specifically selected for producing membrane proteins, and optimizing the bacterial culture growth conditions.

Studies involving molecular chaperones have shown that, in specific cases, chaperones can be very effective for improving monomeric and multimeric recombinant protein assembly. The co-overexpression of the molecular chaperone, GroESL, has been effective in improving production of certain proteins and may be effective for improving the production of yeast SDH in *E. coli*. It should also be feasible to coexpress the $tRNA^{(AGG/AGA)}$, GroESL, and yeast SDH operon genes in one *E. coli* cell by utilizing different selection markers on each plasmid.

Secondly, expression may be tried in various host strains. A mutant strain, C43(DE3), has been specifically selected for its superior ability to overexpress membrane proteins that were shown to be toxic to other expression host strains (96). This strain may have an increased ability to produce yeast SDH.

Optimizing the growth conditions can lead to improved yield of target protein expression. Relevant growth conditions include the fermentation temperature and required nutrient supplements. Temperature is known to have an effect on the expressed recombinant protein. Lower temperatures can support proper processing resulting in improved protein production. In some cases, a lower growth temperature in itself is sufficient to avoid the formation of inclusion bodies or cellular proteolytic degradation. The anaerobic expression of the synthetic yeast SDH operon was studied at 37 °C. I would recommend trying expression at 20 °C, 25 °C, and 30 °C. Aerobic expression of yeast SDH, under the control of the T7 promoter, was tested at 30 °C and resulted in the accumulation of yeast SDH subunits in an insoluble form. This temperature could be reduced to 20 °C. Optimal growth conditions sometimes require the addition of nutrients to the medium. For the homologous overproduction of the NADH dehydrogenase fragment of Complex I, which has 1 FMN and up to 6 iron-sulfur clusters, the addition of riboflavin, sodium sulfide, and ammonium citrate to the culture medium resulted in a reported 60% increase in protein production (15). The overproduction of nNOS required the addition of heme and flavin precursors in addition to the coexpression of a molecular chaperone (115). During homologous overproduction of the *E. coli* SDH, the glycerol-fumarate media was supplemented with 0.035% (wt/vol) tryptone and yeast extract to reduce the lag phase (93). While it has been shown that *E. coli* is able to produce high levels of *E. coli* FRD (86) and *E. coli* SDH (93) under anaerobic growth conditions without the addition of heme or flavin precursors, it may be required for the efficient production of yeast SDH.

A third growth condition to be considered is anaerobic versus aerobic expression. The synthetic yeast SDH operon has been tested under both conditions. Based on the success of expressing both *E. coli* SDH and FRD anaerobically, I would focus on optimizing expression of the synthetic yeast SDH operon anaerobically.

The correct combination of the conditions listed above that alllows for a high-level of expression of the yeast SDH operon will have to be determined empirically.

Finally, an alternative approach to overcoming unknown impediments in expressing the yeast SDH operon is to employ a functional screening assay. Maklashina *et al.* (93) demonstrated that overproduced *E. coli* SDH was capable of complementing growth on glycerol-fumarate anaerobic media in a FRD/SDH double mutant. It is reasonable to predict that the yeast SDH may be able to functionally replace the *E. coli* SDH *in vivo*. Thus, a less-directed approach to elucidating any necessary changes to the synthetic yeast SDH operon to allow for expression, is to create random mutants of the SDH operon and select for expression by growth on glycerol-fumarate medium under anaerobic conditions.

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